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(54) HETEROGENOUS SPECIFIC BINDING ASSAY METHOD AND TEST SYSTEM FOR USE THEREIN

(71) We, MILES LABORATORIES INC., a Corporation organised and existing under the laws of the State of Indiana, United States of America, of 1127 Myrtle Street, Elkhart, Indiana 46514, U.S.A., do hereby declare the invention for which we pray that a Patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to methods and test systems for determining the presence of a ligand in a liquid medium based on the affinity of the ligand for a specific binding partner thereof. In particular, this invention relates to methods and test systems for use in specific binding assays which do not employ radioactive materials or modified enzymes as the labeling substance.

The desirability of a convenient, reliable, and non-hazardous means for detectng the presence of low concentrations of substances in liquids is self-evident. This is particularly true in the field of clinical chemistry where constituents of body fluids which may appear in concentrations as low as 10^{-11} molar are known to be of pathological significance. The difficulty of detecting such low concentrations is compounded in the field of clinical chemistry where sample size is usually quite limited.

Classically, substances have been detected in liquids based on a reaction scheme wherein the substance to be detected is a necessary reactant. The presence of unknown is indicated by the appearance of a reaction product or the disappearance of a known reactant. In certain instances, such an assay method may be quantitative, based on a measurement of either the rate of appearance of product or disappearance of reactant or measurement of the aggregate amount of product produced or reactant consumed in attaining equilibrium. Each assay reaction system is necessarily either limited to use in the detection of only a small group of substances or is non-specific.

The search for assay systems which are highly specific yet adaptable to the detection of a wide range of substances has evolved the radioimmunoassay. In this system a known amount of a radiolabeled form of the substance to be detected is allowed to compete with the unknown for a limited quantity of antibody specific for the unknown. The amount of the labeled form that becomes bound to antibody varies inversely with the level of unknown present. Inherent in the radioimmunoassay technique is the need to separate the labeled form of substance to be detected which becomes bound to antibody, the bound-phase, from that which does not become so bound, the free-phase. While various ways of accomplishing the required separation have been developed, as exemplified in U.S. Patents Nos. 3,505,019; 3,555,143; 3,646,346; 3,720,760; and 3,793,445, all require at least one separate manipulative step, such as filtering, centrifuging, washing, or draining a column to ensure efficient separation of the bound and free phases. Such separation is often accomplished by forming a system comprised of an insoluble portion containing the bound-phase and a liquid portion containing the free-phase such that the amount of radioactive label in either portion is a function of the extent of binding of the labeled material, and thus a function of the amount of ligand in the sample tested. The term "heterogenous" as generally used by the scientific community and as applied herein, means those specific binding assays wherein a separation of the bound- and free-phases is accomplished. Such a separation is necessary to carry out a specific binding assay where the labeled material in the bound-phase is indistinguishable from that in the free-phase.

Because of the hazard and difficulty of handling radio-active materials, there

have been many attempts to devise convenient specific binding assay systems which are as sensitive and rapid as radioimmunoassays but which utilize features other than radioactivity as the means for monitoring the binding reaction. As will be discussed more fully hereinafter, materials which have been utilized as the labeling substance in place of radioactive atoms or molecules includes such diverse materials as enzymes, fluorescent molecules, and bacteriophages.

Exemplary of methods which have been developed using an enzyme as the labeling substance are those described in U.S. Patents Nos. 3,654,090; 3,791,932; 3,839,153; 3,850,752; and 3,879,262 and in the *Journal of Immunological Methods 1*: 247 (1972) and the *Journal of Immunology 109*: 129 (1972). In each of the described methods an enzyme is chemically coupled to either the ligand to be detected or a binding partner thereof and an appropriate heterogenous specific binding reaction scheme is constructed whereby after incubation with a sample, the amount of enzymatic activity associated with either the insoluble portion or the liquid portion is a function of the amount of ligand in the sample. The problems associated with the synthesis and characterization of the enzyme-conjugates are serious short comings of this approach.

Of interest is the enzyme-tagged immunoassay described in U.S. Patent No. 3,817,837. This method does not require the use of a partitioned (i.e. insoluble portion/liquid portion) specific binding reaction system and the separation procedure necessitated thereby since the enzyme-tagged ligand is designed such that upon reaction with the binding partner of the ligand, enzymatic activity is inhibited. Thus, the ratio of bound tagged material to that in free form can be determined by monitoring changes in enzymatic activity. Nonetheless, this method suffiers from the difficulty of preparing well-characterized enzyme-tagged conjugates and of finding enzymes that will fit the basic design of the system.

British Patent No. 1,392,403 and French Patent No. 2,201,299 describe a specific binding assay which utilizes a non-active precursor of a spectrophotometrically-active substance as the labeling substance. After incubation of the sample with the specific binding reaction system, the insoluble and liquid portions are separated and the amount of labeling substance present in the liquid portion, which is a function of the amount of ligand to be detected in the sample, is determined by carrying out reaction steps that transform the inactive labelling substance into a chromogen or fluorometrically active material which is then measured by conventional means.

Other specific binding assay methods employing different types of labeling substances are disclosed in: U.S. Patent No. 3,850,578 which discloses the use of electron spin resonance as a labeling means; U.S. Patent No. 3,901,654 which discloses the use of fluorescence quenching and enhancement as a labeling means; and Report No. PB-224,875 of the National Technical Information Service (NTIS) of the United States Department of Commerce (1973) which describes an unsuccessful attempt to use hemin chloride as a labeling substance in a heterogenous assay system monitored by a chemiluminescence reaction. *Nature 219*: 186 (1968) describes in great detail certain radioimmunoassay procedures and makes a passing reference of a very general nature to the possible use of coenzymes and viruses in place of radioisotopes as labeling substances. However, the author provides no enlightenment as to how to carry out an assay using such alternative labeling substances, or in fact as to whether such an assay would be operable. For further background, reference may be had to *Principles of Competitive Protein-Binding Assays*, ed. Odell and Daughaday (J. B. Lippincott Co., Philadelphia, 1972) which discusses in breadth the various known assay schemes and the different materials and features that have been used as labels for specific binding assays.

Even though many new types of specific binding assays have been suggested and investigated, the radioimmunoassay and the various enzyme-tagged immunoassays remain the most widely used and improved. However, both types of systems have obvious shortcomings, the radioimmunoassay in its use of radioactive material which is hazardous and requires careful handling and the enzyme-tagged immunoassays in the difficulty of preparing useful enzyme-tagged conjugates.

The present invention provides a novel method and test system for detecting a ligand in a liquid which do not employ inconvenient radioactive materials or modified enzymes as the labeling substance.

The present invention provides a highly convenient, versatile, and sensitive improved heterogenous specific binding assay method and test system based on the use of as labelling substance, a substance of the classes hereinafter defined which exhibits reactant activity as a constituent of a predetermined reaction, such substance being referred to herein as the reactant.

The heterogenous specific binding method of the present invention for assaying a liquid medium for a ligand comprises:

(a) contacting the said liquid medium with reagent means comprising a labelled conjugate of (1) a labelling substance having a predetermined activity as (i) a substrate in an enzyme-catalysed chemical reaction, (ii) a reactant in a cyclic chemical reaction, (iii) a reactant in a chemiluminescent reaction, or (iv) a coenzyme in a chemical reaction, and (2) a binding component, the said reagent means forming with the said ligand a binding reaction system so as to produce a bound-phase in which the binding component of the said labelled conjugate is bound by a specific binding partner therefor and a free-phase in which the binding component of the labelled conjugate is not bound by a specific binding partner therefor, the amount of the predetermined characteristic of the said labelling substance in said bound-phase or the said free-phase being a function of the amount of said ligand present in said liquid medium under assay;

(b) separating the said bound-phase from the said free-phase; and

(c) determining the quantity of the said labelling substance present in either the said bound-phase or the said free-phase, by respectively (i) adding the said enzyme thereto and measuring the substrate activity of the labelling substance therein, (ii) forming the said cyclic chemical reaction therein and measuring the said activity, (iii) forming said chemiluminescent reaction therein and measuring the light produced, or (iv) forming the enzyme reaction therein and measuring the said coenzyme activity.

The monitoring reaction system preferably is enzyme-catalyzed. Usually, a monitoring reaction system is selected which is highly sensitive to the reactant in the conjugate. Luminescent or fluorescent reaction systems are very useful in this regard. Particularly preferred are cyclic reaction systems, especially those in which the reactant is the cycled material. Of the preferred cyclic reaction systems, those which are enzyme-catalyzed are particularly advantageous. The reactant in the conjugate is usually an enzymatic reactant, such as an enzyme substrate or, as is particularly preferred, a coenzyme, and preferably has a molecular weight of less than 9000.

In the context of this disclosure, the following terms shall be defined as follows: ligand is the substance, or group of substances, whose presence or the amount thereof in a liquid medium is to be determined; specific binding partner of the ligand is any substance, or group of substances, which has a specific binding affinity for the ligand to the exclusion of other substances; and specific binding analog of the ligand is any substance, or group of substances, which behaves essentially the same as the ligand with respect to the binding affinity of the specific binding partner for the ligand.

The specific binding reagent means may take many different forms. In general, such means comprises three basic constituents, which are (1) the ligand to be detected, (2) a specific binding partner of the ligand, and (3) a labeled constituent which is normally a labeled form of (a) the ligand, (b) a specific binding analog of the ligand, or (c) the specific binding partner. The binding reaction constituents are combined simultaneously or in a series of additions, and with an appropriate incubation period or periods the labeled constituent becomes bound to its corresponding competing binding partners such that the extent of binding, i.e. the ratio of the amount of labeled constituent bound to a binding partner to that unbound, is a function of the amount of ligand present. To follow is a brief description of some of the different binding reaction schemes that may be used in carrying out the method of the present invention.

While in conventional heterogenous specific binding assay methods, such as radioimmunoassays and heterogenous enzyme immunoassays, the labeling characteristic in the labeled conjugate, such as radioactivity or enzymatic activity, is essentially the same for the bound- and free-forms of the conjugate, according to the present method, the activity of the reactant, as labeling substance, is in certain cases affected by binding of the labeled conjugate. In such a situation the monitoring reaction exhibits a relatively constant character where the ligand is absent from the liquid medium, or is present in an insignificantly small amount. When the ligand is present in the liquid medium, a characteristic or property of the monitoring reaction is altered. Generally, the activity of the conjugated reactant is the extent or rate at which the reactant is capable of participating in the monitoring reaction. Thus, the character of the monitoring reaction is altered by the presence of the ligand in the liquid medium, usually with respect to either the aggregate reaction rate thereof or the equilibrium quantity of one or more reaction products produced thereby.

Usually, in this situation, the ability of the conjugated reactant to participate in the monitoring reaction is decreased upon reaction between the specific binding substance to which it is conjugated and a specific binding counterpart of such specific binding substance, that is, the conjugate in its free state is more active in the monitoring reaction than in its bound state.

For the diagrams which are set out hereinafter, the following legend shall apply:

Symbol	Definition
L	ligand to be detected
Ⓛ	ligand or specific binding analog thereof
B	binding partner for the ligand
*	labeling substance, i.e. reactant
├	insoluble phase
→	incubation period followed by appropriate separation
(lim)	limited; present in an amount less than that capable of being bound to the total available binding sites under the selected reaction conditions during the selected incubation period; i.e. the constituent for which the other constituents compete for binding
(exc)	excess, present in an amount greater than that capable of being bound by the total available binding sites under the selected reaction conditions during the selected incubation period

Heterogenous Assay Schemes

1. Competitive binding formats
   a) L+Ⓛ*+B(lim)→+insolubilizing agent for B or Ⓛ*
   This is the classical competitive binding approach. Examples of such insolubilizing agents are specific precipitating antibodies, specific insolubilized antibodies, and, where B or Ⓛ* is a proteinaceous material protein precipitators such as ammonium sulfate, or where B or Ⓛ* is a small absorbable molecule, dextran-coated charcoal. Description of parallel systems may be found in *Biochem. J. 88*:137 (1963) and U.S. Patent No. 3,839,153.
   b) L+Ⓛ*+├ B(lim)→
   This approach is commonly referred to as the solid-phase technique. Descriptions of parallel radioimmunoassay and enzyme immunoassay techniques may be found in U.S. Patents Nos. 3,505,019; 3,555,143; 3,646,346; and 3,654,090.
   c) L+B*+├ Ⓛ(lim)→
   Reference: U.S. Patent No. 3,654,090.
   d) L+├ Ⓛ+B*(lim)→
   Reference: U.S. Patent No. 3,850,752.

2. Sequential saturation formats
   a) L+B(exc)→+Ⓛ*(exc)→+insolubilizing agent for B or Ⓛ*
   In the sequential saturation technique, some or all the binding sites on B remaining after the first incubation period are bound by the labeled constituent.
   b) L+├ B(exc)→+Ⓛ*(exc)→
   Descriptions of parallel radioimmunoassay and enzyme immunoassay techniques may be found in U.S. Patent No. 3,720,760 and *J. Immunol. 209*:129 (1972).
   c) L+B*(exc)→+├ Ⓛ(exc)→

3. "Sandwich" format
   L+├ B(exc)→B*(exc)→
   In the sandwich technique, some or all of the ligand molecules bound to the insolubilized binding partners are bound by the labeled constituent.
   Reference: U.S. Patent No. 3,720,760.

4. Solid-phase dilution format
   L+Ⓛ*+├ (nonspecific)→+B(lim)→
   In this technique, the ligand and the labeled constituent are bound to a nonspecific binder and thereafter proportional amounts are dissociated therefrom by binding with a binding partner having a greater affinity for the

ligand and the labeled constituent. The most useful form of this technique employs a column of the non-specific binder as described in U.S. Patent No. 3,659,104. Such a technique is useful where the ligand is bound to endogenous binding substances in the sample which unless removed would interfere with the competitive binding reaction. Upon being bound to the non-specific binder, the endogenous binding substances may be removed by appropriate washes.

For further discussion of the parameters involved in conventional heterogenous assay systems, such as more detailed descriptions of assay formats and alternative separation techniques, reference may be had to *Principles of Competitive Protein-Binding Assays,* ed. Odell and Daughaday (J. B. Lippincott Co., Philadelphia, 1972).

It is contemplated that manipulative schemes involving other orders of addition and other binding reaction formats may be devised for carrying out heterogenous specific binding assays without departing from the inventive concept embodied herein.

The step of assessing the activity of the conjugated reactant as a constituent of the predetermined monitoring reaction system in either of the bound- or free-phases is conveniently accomplished by contacting such phase with at least one substance which forms with the conjugated reactant, the monitoring reaction, and measuring a characteristic of such reaction. The monitoring reaction system may comprise a simple chemical transformation or a plurality or series of chemical transformations.

Where an enzyme-catalyzed reaction system is used, it includes, in addition to the conjugated reactant, at least one enzyme and may include one or more enzymatic reactants such as substrates and coenzymes. Such enzyme-catalyzed reaction system may comprise a single simple enzymatic reaction or a complex series of enzymatic and non-enzymatic reactions. For instance, the enzyme-catalyzed reaction system may consist of a single enzyme-catalyzed degradation or dissociation reaction. In such a system, the conjugated reactant is the enzyme substrate which undergoes degradation or dissociation, and the only component of the reaction system necessary to be contacted with the selected bound- or free-phase is an enzyme which catalyzes the degradation or dissociation reaction. A more complex enzyme-catalyzed reaction system may consist of a single enzymatic reaction involving two or more reactants or may consist of a series of reactions involving several reactants, at least one of which reactions is enzyme-catalyzed. In such a system, the conjugated reactant would be one of the enzymatic reactants in the enzyme-catalyzed reaction and the selected bound- or free-phase would be contacted with the appropriate enzyme and reactant constituents, other than that in the conjugate, necessary to provide the selected enzyme-catalyzed reaction system.

It is further contemplated that the enzyme-catalyzed reaction system may comprise a biochemical system as complex as the metabolic system of a biological cell such as a microorganism. For example, a nutrient substance essential to the growth of a particular microorganism may be selected as the reactant in the conjugate. Reactant activity would be measurable by monitoring a characteristic of the microorganism, such as the rate of microorganism growth, when such microorganism would be placed in an environment wherein the only source of the reactant nutrient substance is the conjugate.

The appropriate reaction constituents which form, together with the reactant in the conjugate, the monitoring reaction system may be contacted with the selected separated phase mixture singularly or in any combination either prior to, simultaneous with, or subsequent to initiation of the specific binding reaction. After initiation of the specific binding reaction, the reaction mixture, which may include any or all of the necessary components for the monitoring reaction is usually incubated for a predetermined period or periods of time before separation of the resulting bound- and free-phases. After separation, any components which are necessary for the monitoring reaction and which are not already present in sufficient quantities in the selected separated phase are added thereto, and reactant activity therein is assessed as an indication of the presence or amount of the ligand in the liquid medium.

When the reaction rate of the monitoring reaction is the characteristic used to assess reactant activity in the selected bound- or free-phase, as is preferred, such rate is usually determined by measuring the rate of disappearance of a reactant or the rate of appearance of a reaction product. Such measurement can be accomplished by a wide variety of methods including the conventional chromatographic, gravimetric, potentiometric, spectrophotometric, fluorometric, turbidimetric, and volumetric analysis techniques. Since the present method is primarily designed for

the detection of low concentrations of ligands, highly sensitive reaction systems have been developed for use in conjunction with the novel specific binding reaction system.

One preferred form of the monitoring reaction includes a luminescent reaction system, preferably enzyme-catalyzed, such as a reaction exhibiting the phenomenon of bioluminescence or chemiluminescence. The reactant in the conjugate may be a reactant in either the light-producing reaction or a reaction which is preliminary to an enzymatic or non-enzymatic luminescent reaction. The activity of the conjugated reactant can be assessed by following the rate of light production or the total amount, peak intensity, or character of the light produced. Examples of luminescent reaction systems are given in Table A in which the following abbreviations are used:

ATP	adenosine triphosphate
AMP	adenosine monophosphate
NAD	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
FMN	flavin mononucleotide
$FMNH_2$	reduced flavin-mononucleotide
$h\nu$	electromagnetic radiation, usually in the infrared, visible, or ultraviolet region

TABLE A

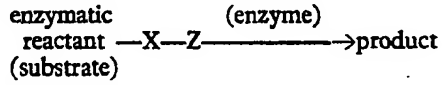
	Luminescent Reaction System	Conjugated Reactant
A.	ATP + reduced luciferin $\xrightarrow[\text{(fire fly)}]{\text{luciferase}}$ $h\nu$ + AMP + oxidized luciferin	ATP or reduced luciferin
B.	$FMNH_2$ + long-chain aldehyde + O_2 $\xrightarrow[(P.\ fisheri)]{\text{luciferase}}$ $h\nu$ + FMN + long-chain acid + H_2O	$FMNH_2$ or long-chain aldehyde
C.	1) NADH + FMN + $H^{\oplus}$ $\xrightarrow{\text{NADH dehydrogenase}}$ NAD + $FMNH_2$	NADH or FMN
	2) $FMNH_2$ + long-chain aldehyde + O_2 $\xrightarrow[(P.\ fisheri)]{\text{luciferase}}$ $h\nu$ + FMN + long-chain acid + H_2O	
D.	1) 3',5'-adenosine diphosphate + reduced luciferin sulfate $\xrightarrow[\text{transferase}]{\text{sulfate}}$ adenosine-3'-phosphate-5'-phosphosulfate + reduced luciferin	3',5'-adenosine diphosphate or reduced luciferin
	2) reduced luciferin + O_2 $\longrightarrow$ $h\nu$ + oxidized luciferin	
E.	luminol + H_2O_2 $\xrightarrow{\text{peroxidase*}}$ $h\nu$ + aminophthalate + N_2	luminol
F.	reduced pyrogallol + H_2O_2 $\xrightarrow{\text{peroxidase*}}$ $h\nu$ + oxidized pyrogallol + H_2O	reduced pyrogallol
G.	luminol + O_2 $\xrightarrow{\text{oxygenase}}$ $h\nu$ + aminophthalate + N_2	luminol
H.	reduced pyrogallol + O_2 $\xrightarrow{\text{oxygenase}}$ $h\nu$ + oxidized pyrogallol	reduced pyrogallol
I.	isoluminol + H_2O_2 $\xrightarrow{\text{lactoperoxidase}}$ $h\nu$ + aminophthalate + N_2	isoluminol
J.	isoluminol + KO_2 $\longrightarrow$ $h\nu$ + aminophthalate + N_2	isoluminol

*or catalase

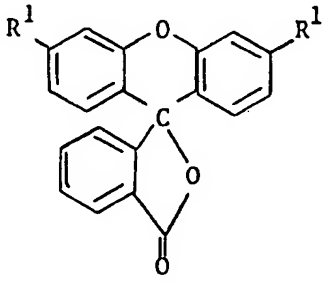
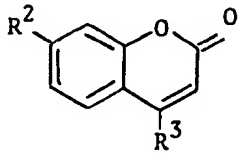
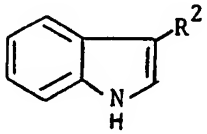
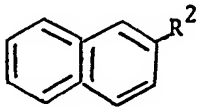
Further details and discussion concerning luminescent reaction systems which may be used in the present method may be found in the following references:

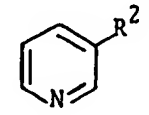
J. Biol. Chem. 236: 48 (1961).
J. Amer. Chem. Soc. 89: 3944 (1967).
Cormier et al., *Bioluminescence in Progress*, ed. Johnson et al., Princeton University Press (New Jersey, 1966) pp. 363—84.
Kries, P. *Purification and Properties of Renilla Luciferase*, doctoral thesis University of Georgia (1967).
Am. J. Physiol. 41: 454 (1916).
Biol. Bull. 51: 89 (1926).
J. Biol. Chem. 243: 4714 (1968).

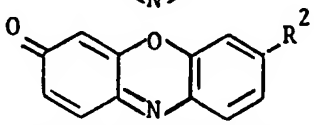
Another type of preferred, sensitive, monitoring reaction involves the phenomenon of fluorescence and is enzyme-catalyzed. In such a reaction system the reactant in the conjugate is a substrate in an enzymatic reaction which produces a product which has a fluorescent property which distinguishes it from the conjugated substrate. A general reaction scheme for such an enzyme-catalyzed reaction system is as follows:



wherein X is an enzyme-cleavable bond or linking group, such as an ester or amido group, and Z is a specific binding substance which, depending upon the specific binding reaction technique used, is the ligand, a specific binding analog of the ligand, or a specific binding partner of the ligand. Specific conjugates which may be used in this type of reaction system are various enzyme-cleavable modifications and derivatives of fluorescein, umbelliferone, 3-indole, β-naphthol, 3-pyridol, and resorufin. Examples of possible structural formulas of such derivatives are as follows:

Derivative	Formula
fluorescein	
umbelliferone	
3-indole	
β-naphthol	

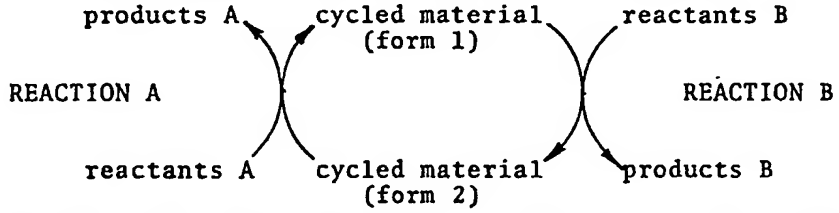
3-pyridol 

resorufin 

wherein R^1 is —OH or —X—Z (as defined above in this paragraph), R^2 is —X—Z, and R^3 is —H or —CH_3.

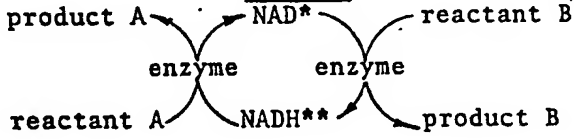
A reaction system which is particularly preferably for use in assessing the activity of the conjugated reactant in the selected separated phase is a cyclic or cycling reaction system. Such a reaction system is one in which a product of a first reaction is a reactant in a second reaction, which second reaction has as one of its products a substance that is also a reactant in the first reaction.

The following diagram illustrates a model of a cyclic reaction system:



In the above model cyclic reaction system, a small amount of cycled material, if provided with sufficient amounts of reactants A and B, will generate large amounts of products A and B. Since the rate and amount of product produced by the reactions constituting the cyclic reaction system is highly sensitive to the amount of cycled material present, it is most preferred to use the cycled material as the reactant in the conjugate of the present invention. Examples of cycling reaction systems contemplated for use in conjunction with the novel specific binding reaction system of the present invention are given in Tables B, C, and D.

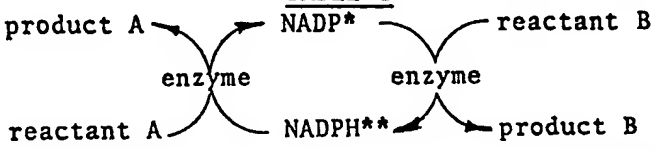
TABLE B



reaction	reactant A or product B	enzyme	reactant B or product A
1	lactaldehyde	alcohol dehydrogenase	propanediol
2	α-ketoglutarate+NH_3	glutamic dehydrogenase	glutamate
3	oxaloacetate	malic dehydrogenase	malate
4	acetaldehyde	alcohol dehydrogenase	ethanol
5	α-ketoglutarate+CO_2	isocitric dehydrogenase	isocitrate
6	dihydroxyacetone phosphate	α-glycerol phosphate dehydrogenase	L-α-glycerol phosphate
7	pyruvate	lactic dehydrogenase	lactate
8	1,3-diphosphoglycerate	glyceraldehyde-3-phosphate dehydrogenase	glyceraldehyde-3-phosphate +phosphate

* nicotinamide adenine dinucleotide
** reduced NAD

TABLE C



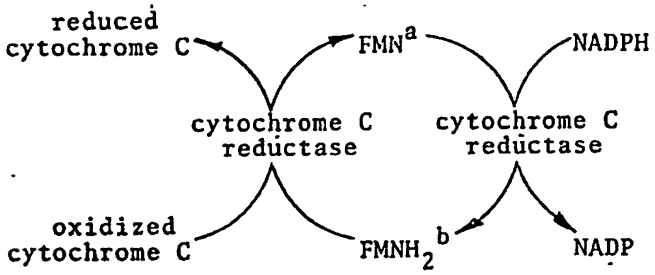
reaction	reactant A or product B	enzyme	reactant B or product A
1	6-phospho-gluconate	glucose-6-phosphate dehydrogenase	glucose-6-phosphate
2	oxidized glutathione	glutathione reductase	reduced glutathione
3	*p*-benzoquinone	quinone reductase	hydroquinone
4	nitrate	nitrate reductase	nitrite
5	α-ketoglutarate + NH_3	glutamic dehydrogenase	glutamate

* nicotinamide adenine dinucleotide phosphate
** reduced NADP

It should be noted that the cyclic reaction systems illustrated in Tables B and C comprise the combination of any one of the reactions listed in the respective tables with any other reaction listed therein. For example, reaction I in Table B may be paired with any one of reactions 2—9 to form a useful cyclic reaction system. Thus Tables B and C represent respectively 56 and 20 possible cyclic reaction systems for use in the present invention.

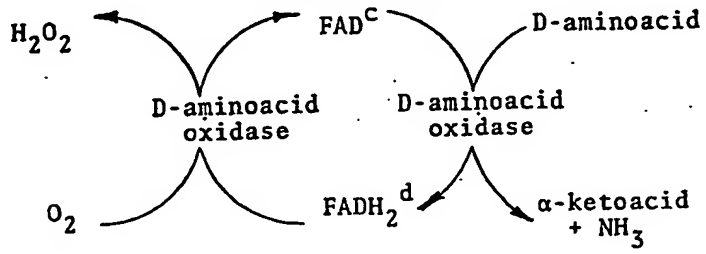
In addition to the cyclic reaction systems represented in Tables B and C, it is contemplated that one of the reactions in the cyclic reaction system may involve the enzymatic or non-enzymatic conversion of a spectrophotometric indicator, preferably colorimetric. An example of a cyclic reaction system involving a conversion of an indicator is the system produced by combining one of the reactant B—product B reactions from Table B with a reaction comprising an oxidation-reduction indicator and an electron transfer agent. As electron transfer agent, phenaziumemethosulfate may be used. Useful indicators include the oxidized forms of nitrotetrazolium, thiazoyl blue, and dichlorophenolindophenol.

TABLE D

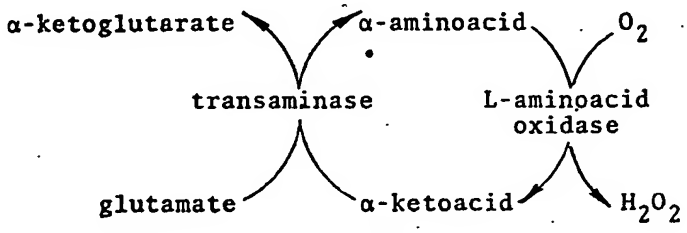


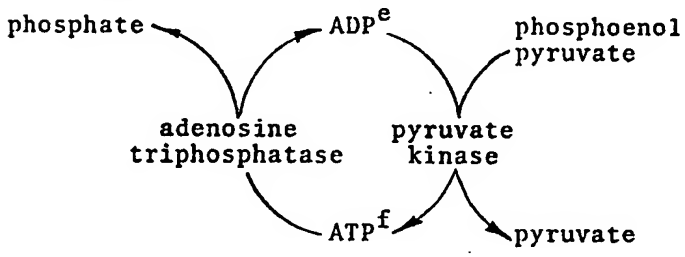
[a] flavin mononucleotide
[b] reduced FMN

TABLE D (cont.)

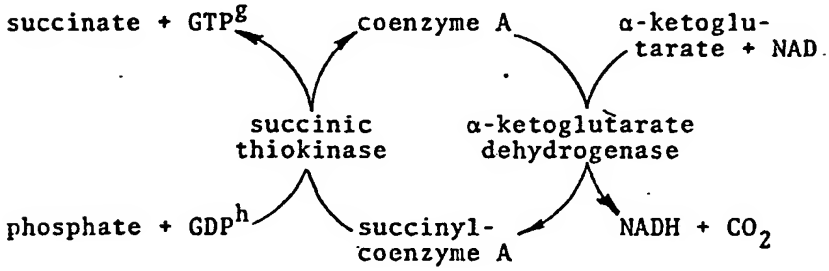


[c]flavin adenine dinucleotide
[d]reduced FAD





[e]adenosine diphosphate
[f]adenosine triphosphate



[g]guanosine triphosphate
[h]guanosine diphosphate

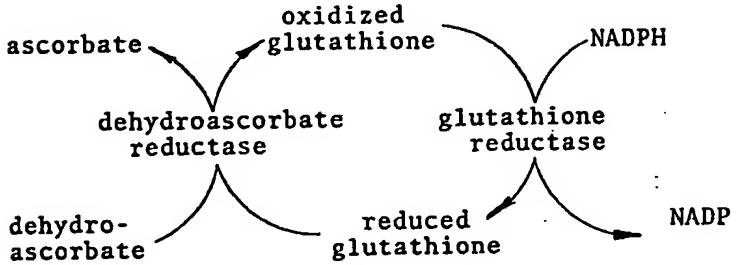
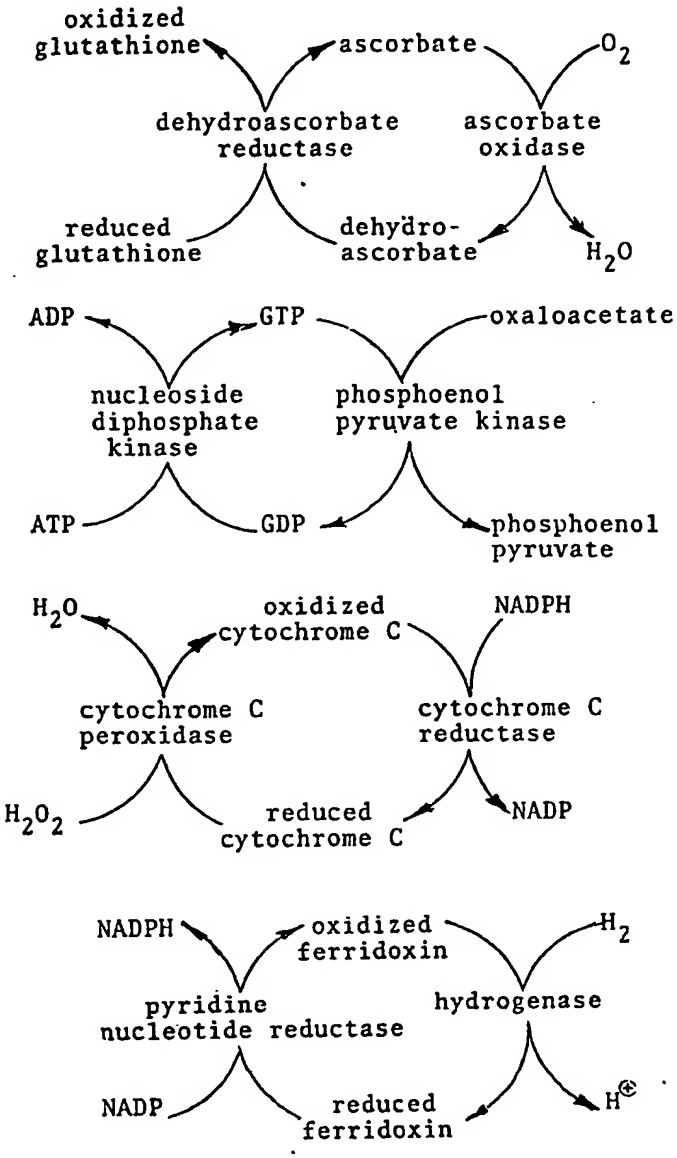


TABLE D (cont.)



In forming any of the cyclic reaction systems illustrated in Tables B, C, and D, where a component in the reaction system is in an ionic form, it may of course be added in a salt or acid form which is ionizable upon contacting the liquid medium. A water soluble salt or acid of such component is usually preferred.

It is also contemplated that an exponential cyclic reaction system may be included in the monitoring reaction system. An example of an exponential cyclic reaction system is as follows:

$$AMP + ATP \xrightarrow{\text{myokinase}} 2\ ADP$$

$$ADP + PEP \xrightarrow{\text{pyruvate kinase}} ATP + \text{pyruvate}$$

Such a cyclic reaction is autocatalytic in the sense that during each cycle the amount of cycled material is doubled. The cycling rate therefore increases exponentially with time and affords a high degree of sensitivity. Further details and discussion relating to such cyclic reactions may be found in *J. Biol. Chem.* 247:3558—70 (1972).

Where a cyclic reaction system is used as a means of assessing any change in activity of the conjugated reactant, the rate of disappearance of a reactant or rate of appearance of a reaction product can be determined by conventional techniques or by using one or more additional cycling systems followed by a conventional determination of the aggregate reaction rate.

The use of a cyclic reaction system in conjunction with the heterogenous specific binding reaction system provides a high degree of assay versatility as well as sensitivity. A single reactant-specific binding substance conjugate may be used with a multiplicity of reactions to form cyclic systems which have sensitivities varying over a wide range and which provide a wide variety of responses detectable by the senses or artificial means. Such versatility is lacking in the prior art.

The present invention may be applied to the detection of any ligand for which there is a specific binding partner. The ligand usually is a peptide, protein, carbohydrate, glycoprotein, steroid, or other organic molecule for which a specific binding partner exists in biological systems or can be synthesized. The ligand, in functional terms, is usually selected from the group consisting of antigens and antibodies thereto; haptens and antibodies thereto; and hormones, vitamins, metabolites and pharmacological agents, and their receptors and binding substances. Specific examples of ligands which may be detected using the present invention are hormones such as insulin, chlorionic gonadotropin, thyroxine, liothyronine, and estriol; antigens and haptens such as ferritin, bradykinnin, prostaglandins, and tumor specific antigens; vitamins such as biotin, vitamin B_{12}, folic acid, vitamin E, and ascorbic acid; metabolites such as 3′,5′ adenosine monophosphate and 3′,5′ guanosine monophosphate; pharmacological agents such as dilantin, digoxin, morphine, digitoxin, and barbiturates; antibodies such as microsomal antibody and antibodies to hepatitis and allergens; and specific binding receptors such as thyroxine binding globulin, avidin, intrinsic factor, and transcobalamine.

In the conjugate of the present invention, the reactant is coupled or bound to a specific binding substance, which is the ligand, a specific binding analog of the ligand, or a specific binding partner of the ligand, depending upon the assay scheme selected, such that a measurable amount of activity of the reactant is retained. The bond between the reactant and the specific binding substance is usually substantially irreversible under the conditions of the assay such as where the predetermined monitoring reaction in which the reactant has activity is not designed to chemically destroy such bond as in the above-mentioned luminescent and cyclic reaction systems. However, in certain instances such bond is by design destroyed or otherwise affected by the selected monitoring reaction as a means for assessing in reactant activity. Such a case is the enzymatic fluorescent substrate reaction systems referred to previously herein.

The reactant may be directly coupled to the specific binding substance so that the molecular weight of the conjugate is less than or equal to the aggregate molecular weight of the reactant and the specific binding substance. Usually, however, the reactant and the specific binding substance are linked by a bridge group comprising between 1 and 50, and preferably between 1 and 10, carbon atoms or heteroatoms such as nitrogen, oxygen, sulfur, or phosphorus. Examples of a bridge group comprising a single atom would be a methylene group (one carbon atom) and an amino group (one heteroatom). The bridge group usually has a molecular weight not exceeding 1000 and preferably less than 200. The bridge group comprises a chain of carbon atoms or heteroatoms, or a combination of both, and is joined to the reactant and the specific binding substance or active derivative thereof, by a connecting group usually in the form of an ester, amido, ether, thioester, thioether, acetal, methylene, or amino group.

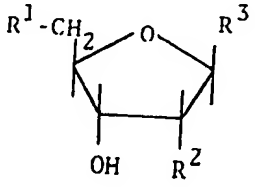
The reactant in the conjugate of the present invention may be any substance which has given (i.e. fixed or known) reactant activity as a constituent of a predetermined monitoring reaction. More particularly, for the purposes of this disclosure, the terms "reactant" and "substance having reactant activity" refer to any chemical substance which is capable of undergoing a finite measurable chemical transformation which yields one or more products different from itself and which results upon interaction of said reactant with reaction-initiating means, such as a chemical substance (i.e. another reactant, a catalyst, or other type ion) electromagnetic radiation, thermal energy, or sonic energy. The class of substances defined herein as "reactants" therefore includes conventional inorganic and organic reagents and various biochemical materials, but excludes such materials as catalysts, including enzymes, and radioactive isotopes which are not reactants in the monitoring reaction. It will be recognized that while a particular chemical substance may be classified in

several different categories because it is able to function in several ways depending on its chemical environment, it is the activity of such substance with respect to the selected monitoring reaction referred to herein which shall govern which functional identity such substance shall have in the context of this disclosure.

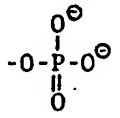
Preferably, the reactant is an enzymatic reactant such as an enzyme substrate, a coenzyme, or an active modification or derivative thereof. An enzyme substrate is a compound or moiety capable of undergoing a chemical transformation that is catalyzed by an enzyme. Where a substrate is employed as the conjugated reactant, the preferred molecular weight thereof is less than 9000 and preferably less than 5000. Substrates of such size, because of their lack of molecular complexity, are most convenient for use in the fabrication of the conjugate. Examples of enzyme substrates which are contemplated for use in the present invention include the enzyme-cleavable fluorescent substrates referred to previously such as fluorescein and umbelliferone derivatives; pH indicators; and spectrophotometric indicator dyes, particularly chromogenic types.

For the above reasons and for reasons of versatility and adaptability, coenzymes are especially preferred for use as the reactant in the conjugate. A coenzyme is a non-protein molecule which migrates from one enzyme protein to another in facilitating the efficient performance of the catalytic function of the enzyme. All known coenzymes have a molecular weight of less than 9000, the preferred coenzymes having a molecular weight of less than about 5000. Useful coenzymes include the nucleotide coenzymes, particularly those comprising adenine groups, such as the adenosine phosphates (i.e. the mono-, di-, and tri-phosphate forms), nicotinamide adenine dinucleotide and its reduced forms, and nicotinamide adenine dinucleotide phosphate and its reduced forms. Other useful coenzymes include the guanosine phosphates, flavin mononucleotide and its reduced forms, flavin adenine dinucleotide and its reduced forms, coenzyme A and its thioesters including succinyl-coenzyme A, 3′,5′ adenosine diphosphate, and adenosine-3′-phosphate-5′-phosphosulfate.

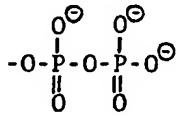
Useful coenzyme-active conjugates comprise nucleotide coenzymes having an adenine group to which the specific binding substance, i.e., a ligand, a specific binding analog of a ligand, or a specific binding partner of a ligand, is coupled through a direct bond or a bridge group as referred to hereinbefore. Such coenzyme-active conjugates which comprise an adenosine phosphate, nicotinamide adenine dinucleotide or its reduced form, or nicotinamide adenine dinucleotide phosphate or its reduced form, have the following general formula:

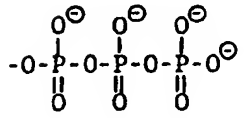


wherein R^1 is

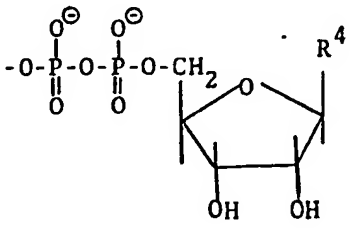


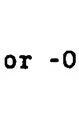
,



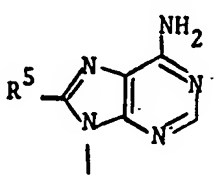
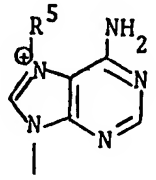
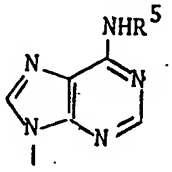
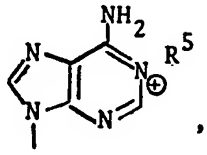
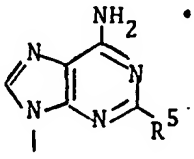
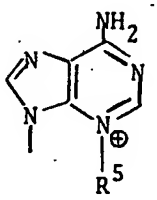


, or

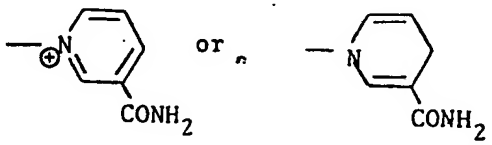


wherein R^2 is -OH or  ;

wherein R^3 is

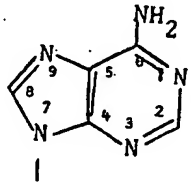







wherein R^4 is



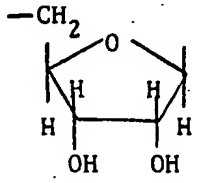
wherein R^5 is —Y—Z; wherein Y is a bond or a bridge group; and wherein Z is a ligand, a specific binding analog of a ligand, or a specific binding partner of a ligand. The above formula represents the ionized forms of the coenzyme-active conjugate, however, the protonized or acid forms are equally useful. The extent of protonization depends on the pH of the environment. Also, the salts of such conjugates may also be used where appropriate.

Synthesis of such compounds may be accomplished in a variety of ways. It is contemplated that the synthesis routes which are schematically illustrated below are advantageously followed in the preparation of the useful compounds. In the illustrated syntheses, the positions on the adenine ring structure are referred to according to the following:

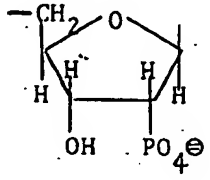


Also, the following abbreviations are used;

Rib refers to the ribose moiety, i.e.,

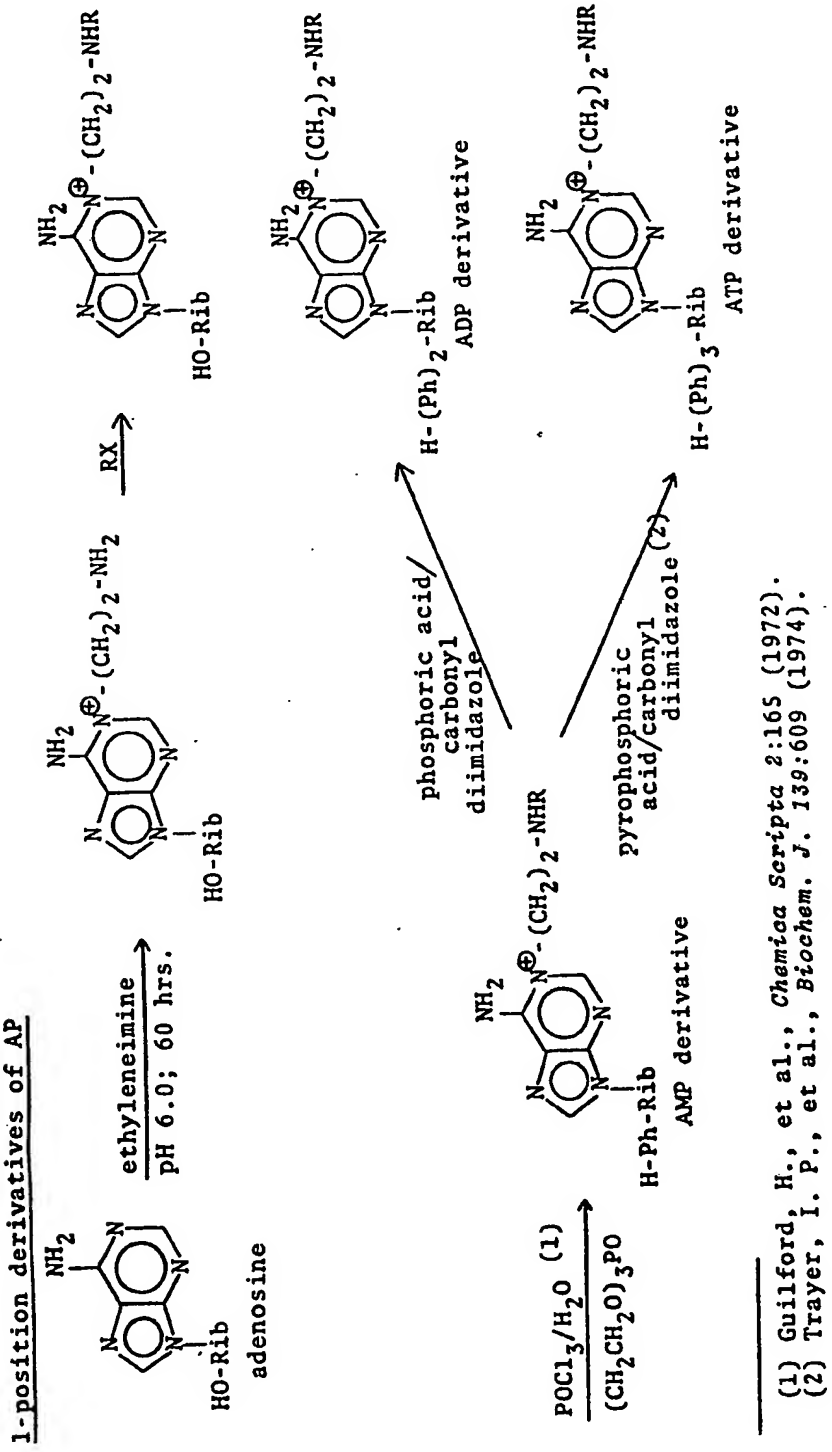


Rib′ refers to the phosphated ribose moiety, i.e.,



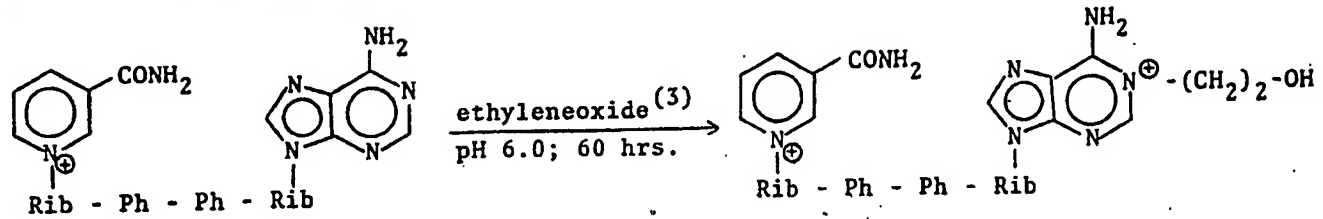
Ph refers to a phosphate group;

AP derivatives refers to derivatives of adenosine-5'-phosphate, i.e., the mono- (AMP), di- (ADP), or tri- (ATP) phosphate form;
NAD derivative refers to a derivative of either nicotinamide adenine dinucleotide or a reduced form thereof;
NADP derivative refers to a derivative of either nicotinamide adenine dinucleotide phosphate or a reduced form thereof;
R refers to the specific binding substance or a modification thereof; and
X refers to a leaving group, usually a halogen.

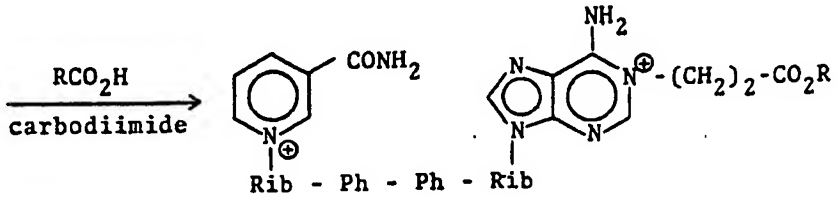


(1) Guilford, H., et al., *Chemica Scripta* 2:165 (1972).
(2) Trayer, I. P., et al., *Biochem. J.* 139:609 (1974).

1-position derivative of NAD



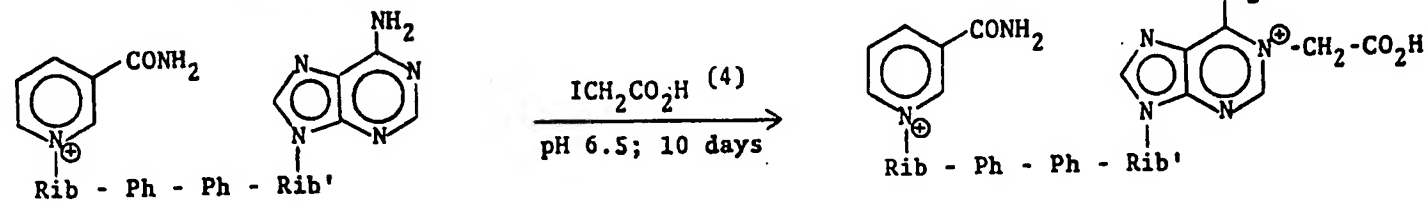
NAD



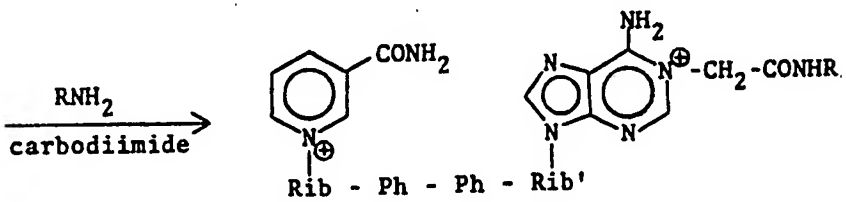
NAD derivative

(3) Windmueller, H.G., and Kaplan, N.O., *J. Biol Chem. 236*:2716 (1961).

1-position derivative of NADP



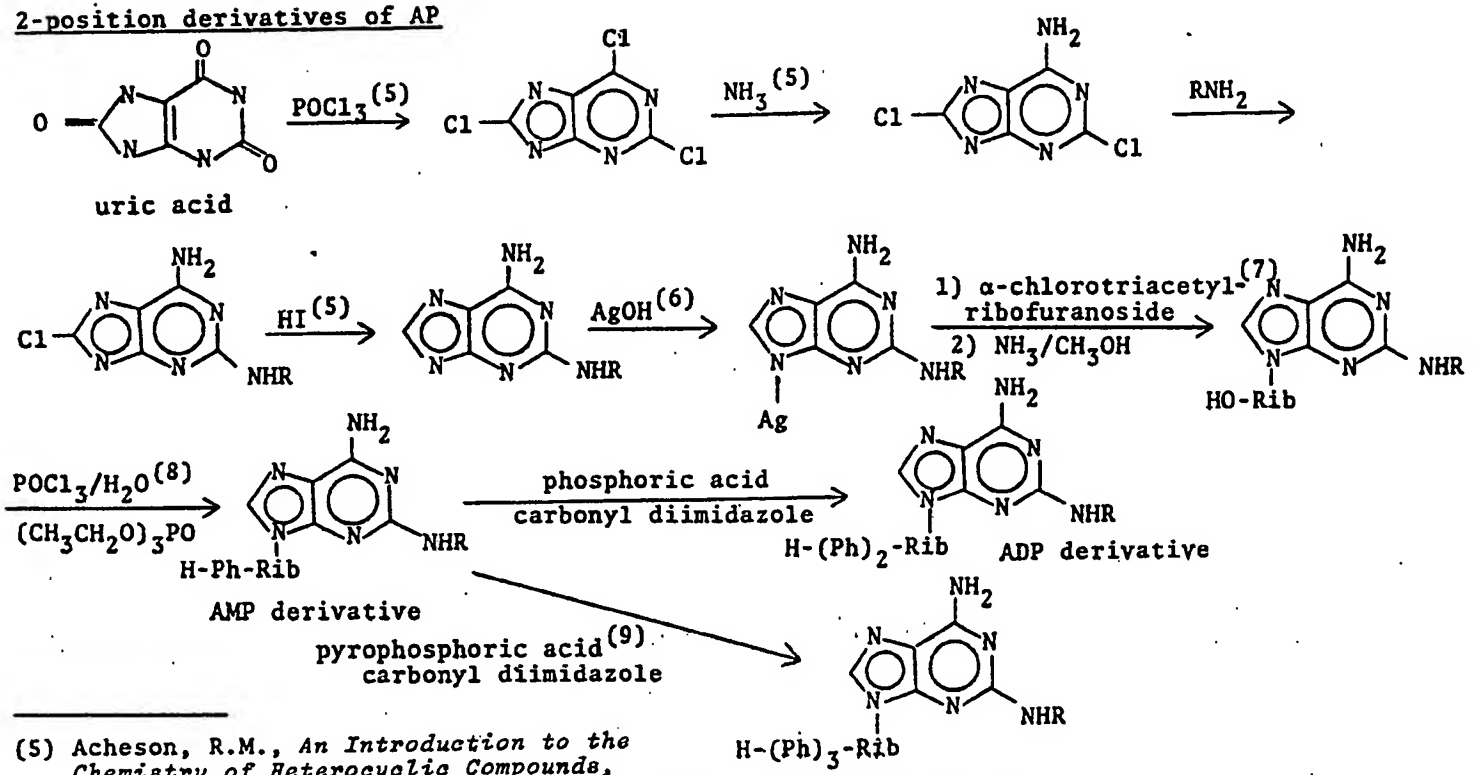
NADP



NADP derivative

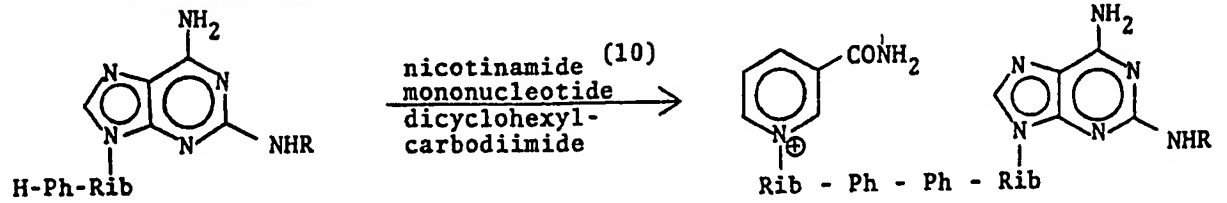
(4) Lowe, C.R. and Mosbach, K., *Eur. J. Biochem.* *49*:511 (1974).

2-position derivatives of AP



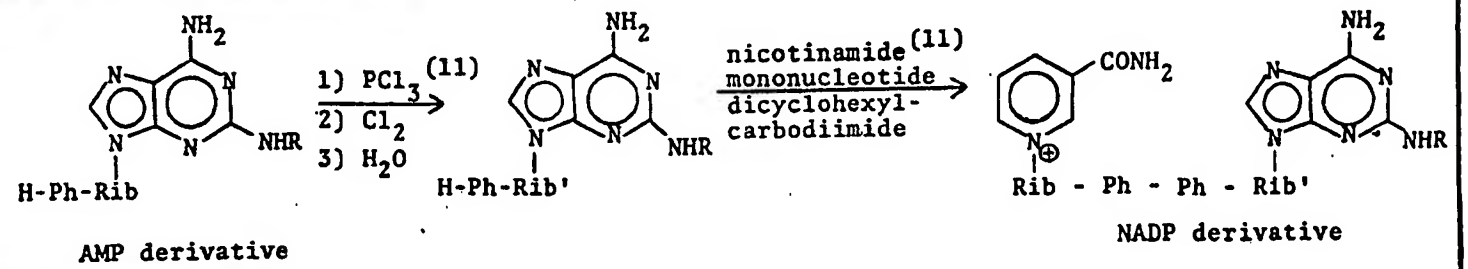
(5) Acheson, R.M., *An Introduction to the Chemistry of Heterocyclic Compounds*, Interscience Publ. (New York 1962), p. 308.
(6) Fischer, E., *Ber.* *30*:2239 (1897).
(7) Davoll et al., *J. Chem. Soc.*, 967 (1948).
(8) Guilford, H., et al., supra.
(9) Trayer, I.P., et al., supra.

2-position derivative of NAD



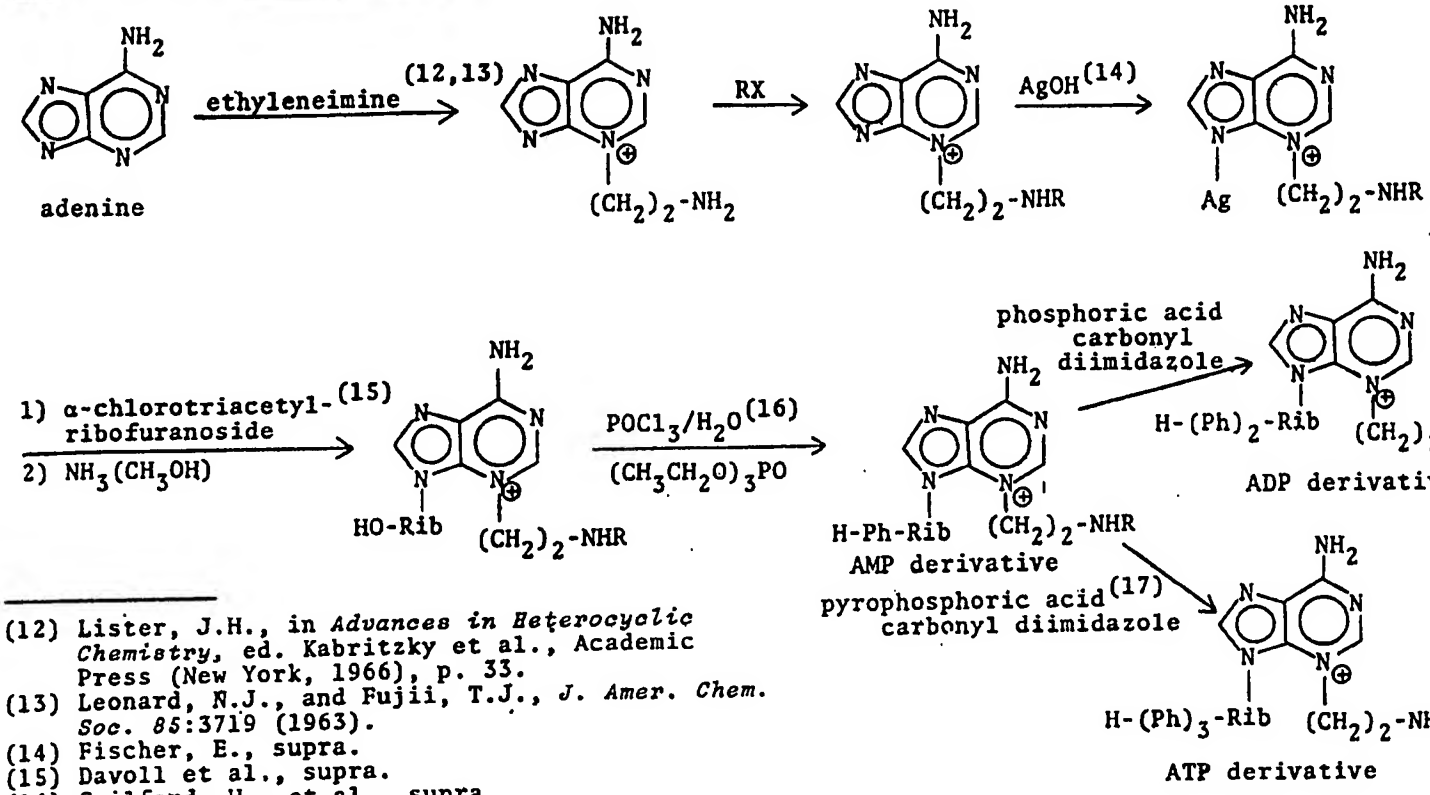
(10) Hughes, N.A., et al., *J. Chem. Soc.*, 3733 (1957).

2-position derivative of NADP



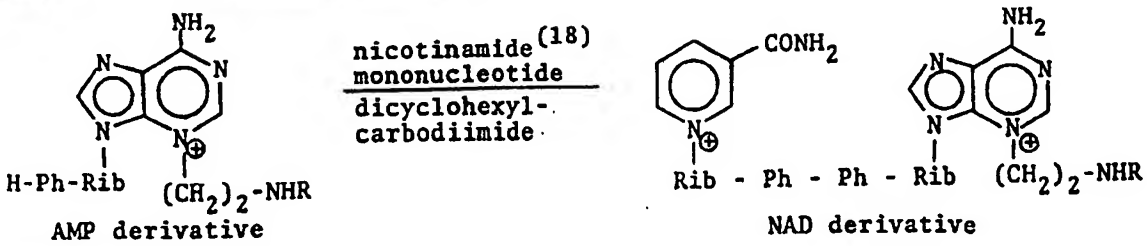
(11) Hughes, N.A., et al., supra.

3-position derivatives of AP



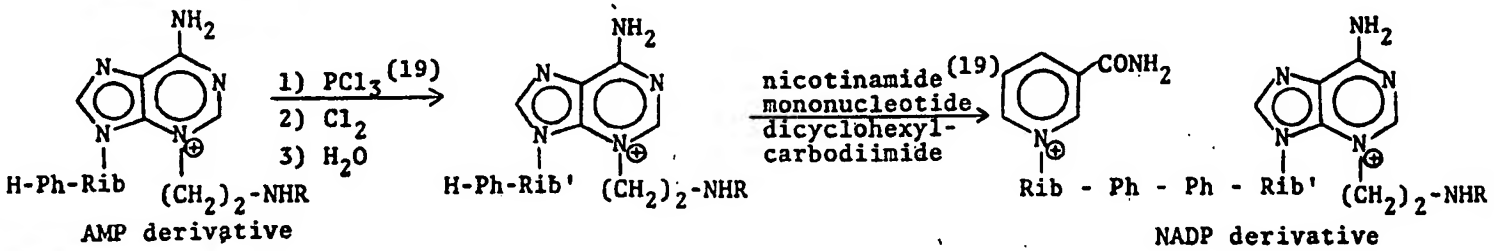
(12) Lister, J.H., in *Advances in Heterocyclic Chemistry*, ed. Kabritzky et al., Academic Press (New York, 1966), p. 33.
(13) Leonard, N.J., and Fujii, T.J., *J. Amer. Chem. Soc.* *85*:3719 (1963).
(14) Fischer, E., supra.
(15) Davoll et al., supra.
(16) Guilford, H., et al., supra.
(17) Trayer, I.P., et al., supra.

3-position derivative of NAD



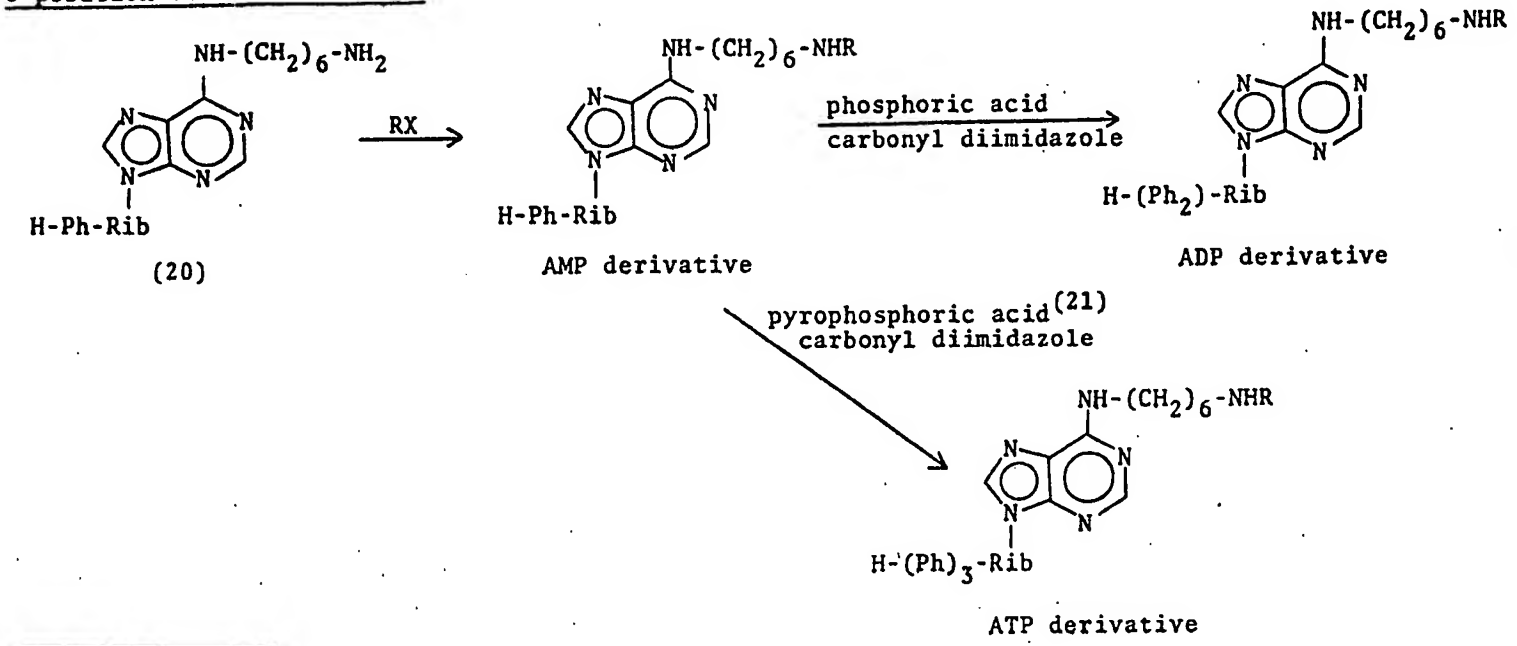
(18) Hughes, N.A., et al., supra.

3-position derivative of NADP



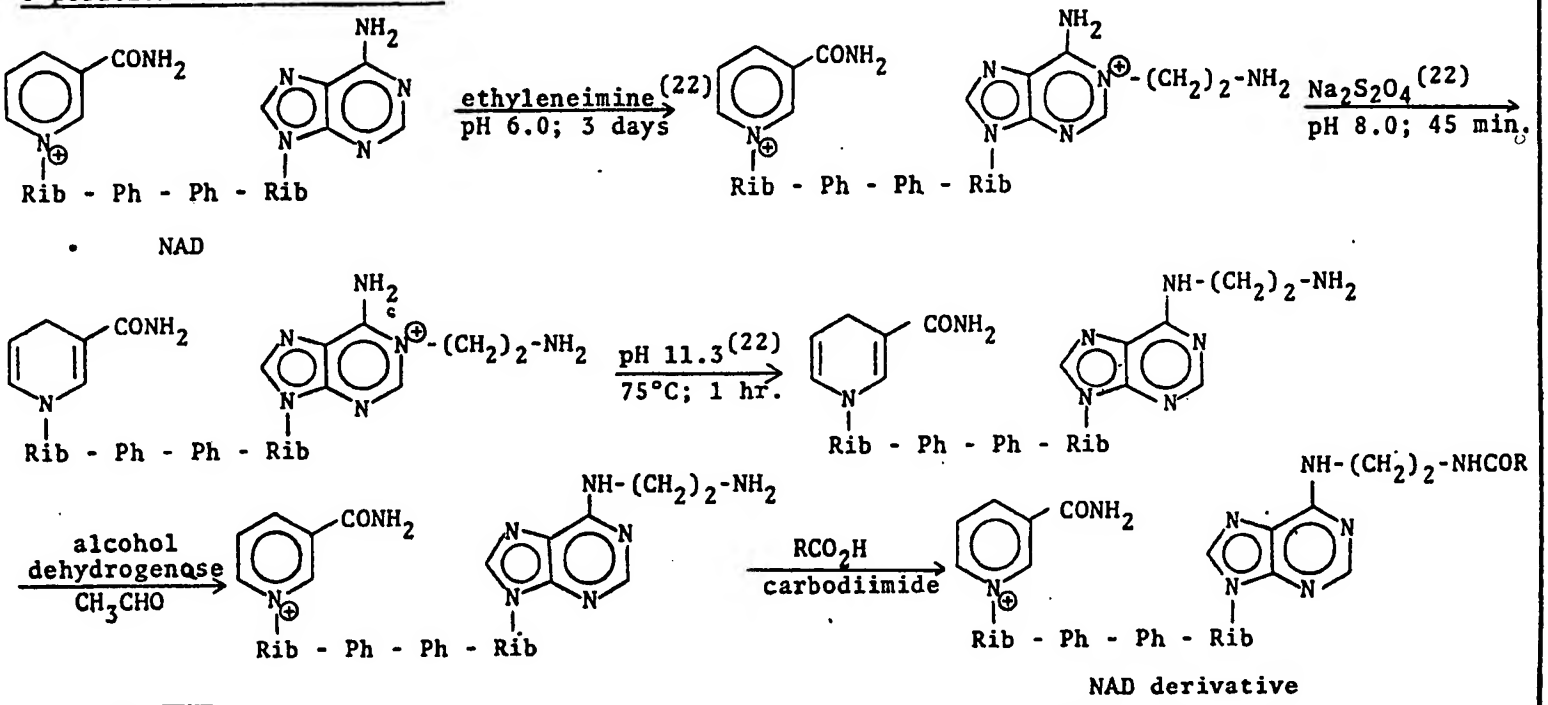
(19) Hughes, N.A., et al., supra.

6-position derivatives of AP



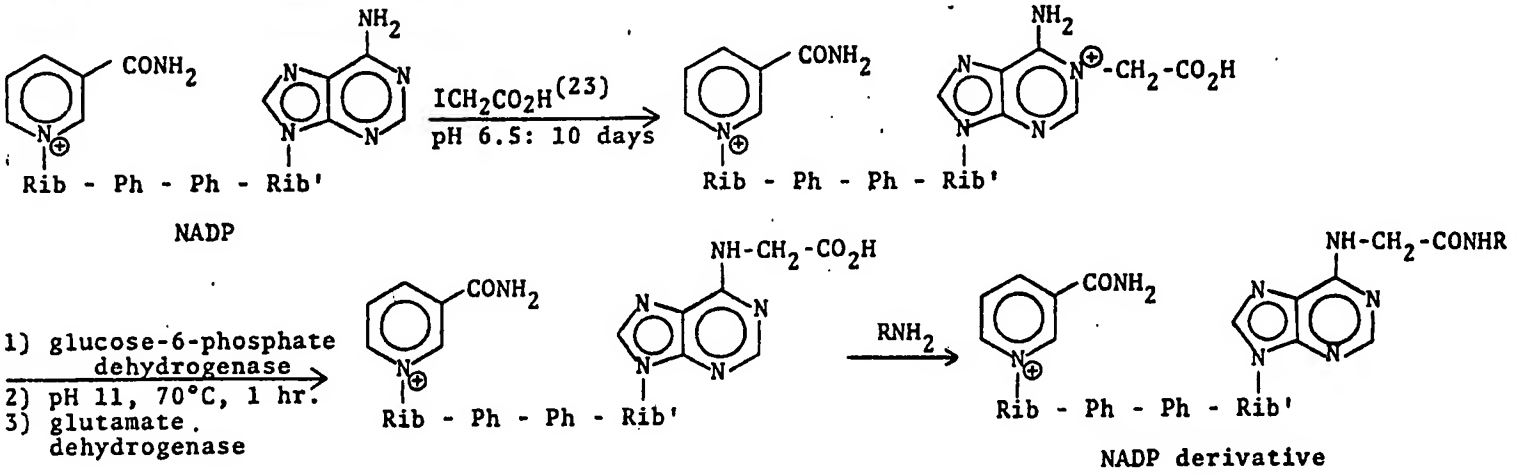
(20) Guilford, H., et al., supra.
(21) Trayer, I.P., et al., supra.

6-position derivative of NAD



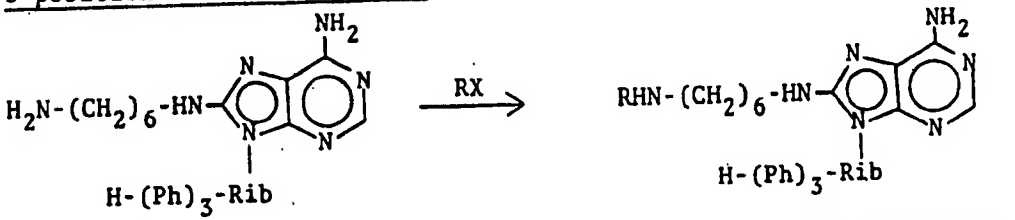
(22) Windmueller, H.G., and Kaplan, N.O., *J. Biol. Chem. 236:*2716 (1961).

6-position derivative of NADP



(23) Lowe, C.R., and Mosbach, K., supra.

8-position derivatives of AP

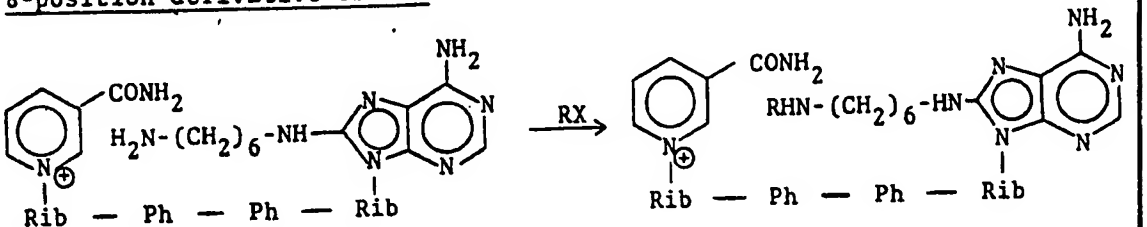


(24)

ATP derivative

(24) Trayer, I.P., et al., supra.

8-position derivative of NAD

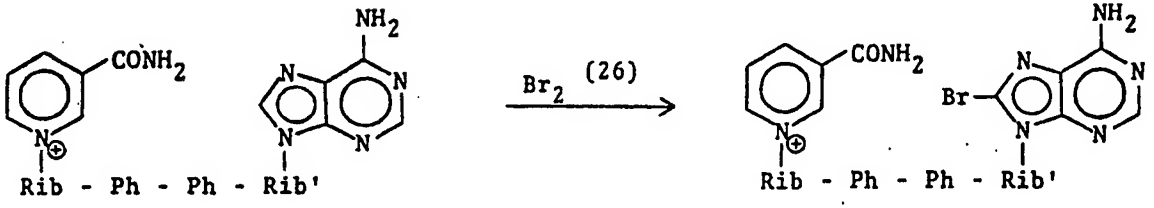


(25)

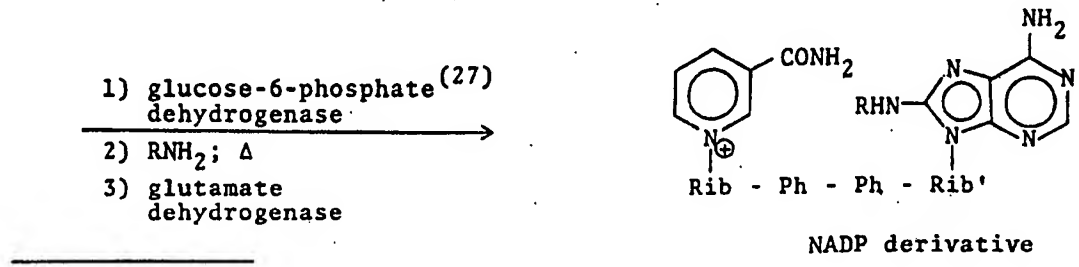
NAD derivative

(25) Lee, C-Y, et al., *Arch. Biochem. Biophys.* *163*:561 (1974).

8-position derivative of NADP



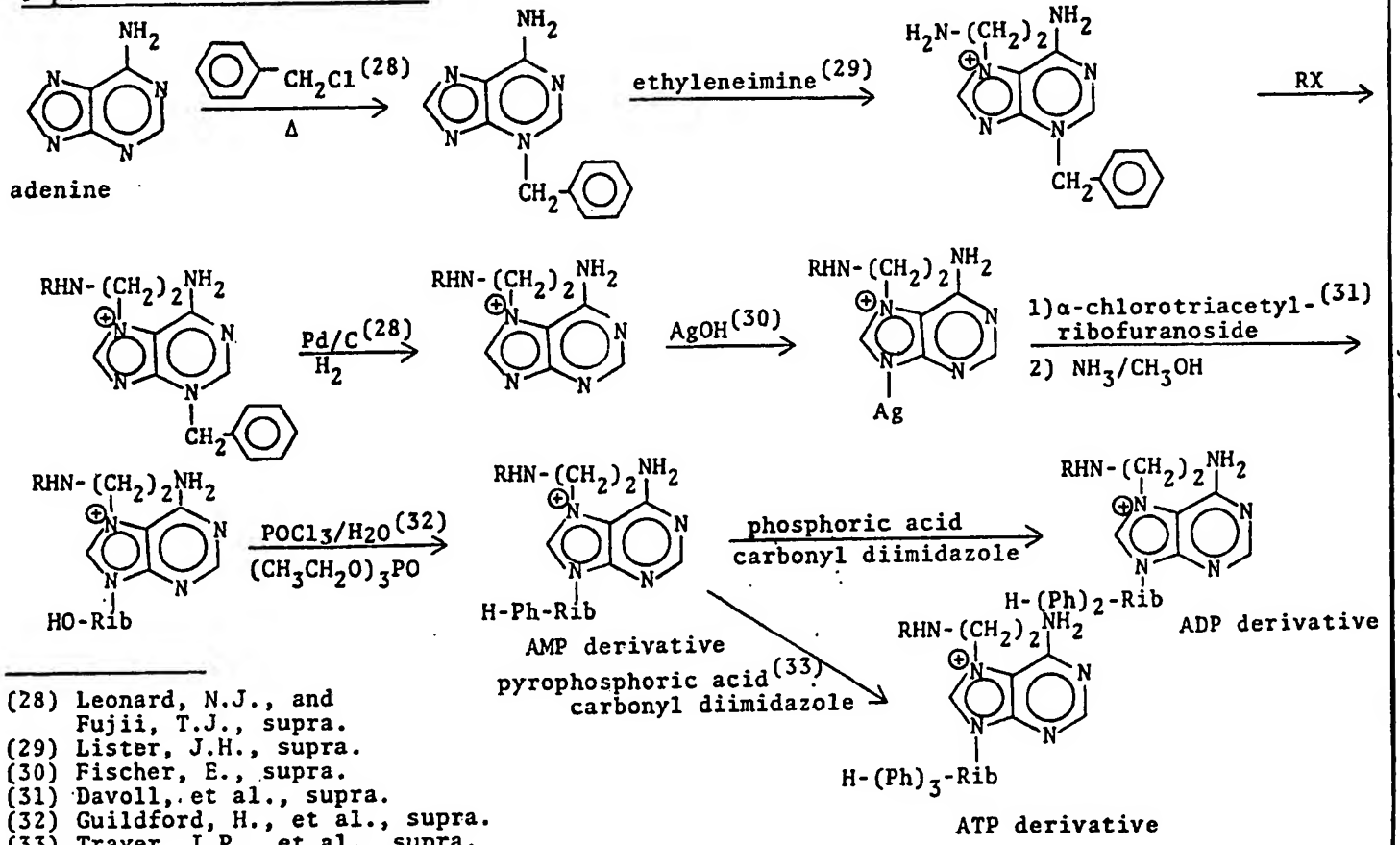
NADP



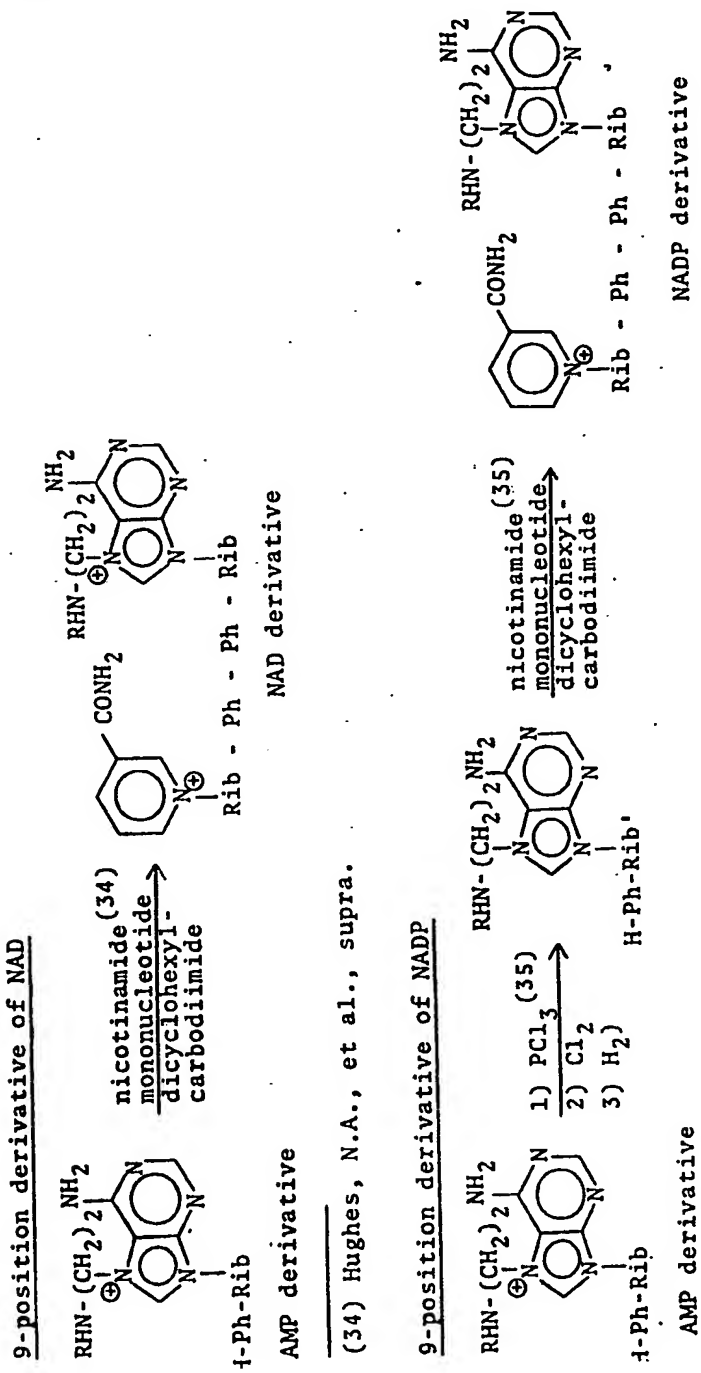
NADP derivative

(26) Lee, C-Y, et al., supra.
(27) Lowe, C.R. and Mosbach, R., supra.

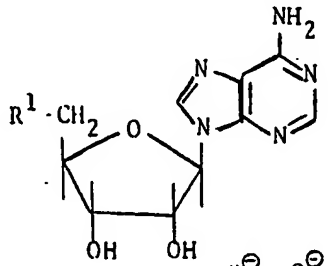
9-position derivatives of AP

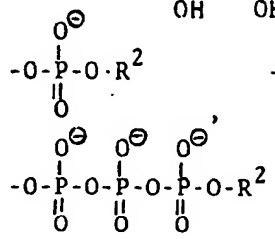
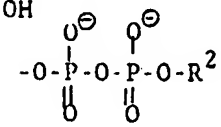


(28) Leonard, N.J., and Fujii, T.J., supra.
(29) Lister, J.H., supra.
(30) Fischer, E., supra.
(31) Davoll, et al., supra.
(32) Guildford, H., et al., supra.
(33) Trayer, I.P., et al., supra.



In addition to the compounds mentioned above, useful coenzyme-active conjugates include the adenosine phosphates to which are coupled the specific binding substance through the phosphate grouping. Such compounds have the following general formula:



wherein R^1 is  or  ,

wherein R^2 is —Y—Z; wherein Y is a bond or a bridge group; and wherein Z is a ligand, a specific binding analog of a ligand, or a specific binding partner of a ligand. Also, the protonized or acid forms as well as the salt forms where appropriate, may be used.

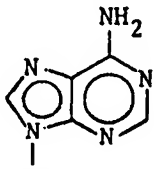
Synthesis of such compounds may be accomplished in a variety of ways. It is contemplated that the synthesis routes which are schematically illustrated below are advantageously followed in the preparation of the useful compounds. The abbreviations used hereinbefore also apply to the illustration to follow.

derivatives of AP

1) $H_2N-(CH_2)_n-OH$ $\xrightarrow[\Delta]{\text{phosphoric acid}}$ $H_2N-(CH_2)_n-Ph-H$ $\xrightarrow{RX}$ $RHN-(CH_2)_n-Ph-H$

n=1-10

$\xrightarrow[\text{carbonyl diimidazole}]{\text{adenosine monophosphate}}$ (36)



$RHN-(CH_2)_n-(Ph)_2-Rib$

ADP derivative

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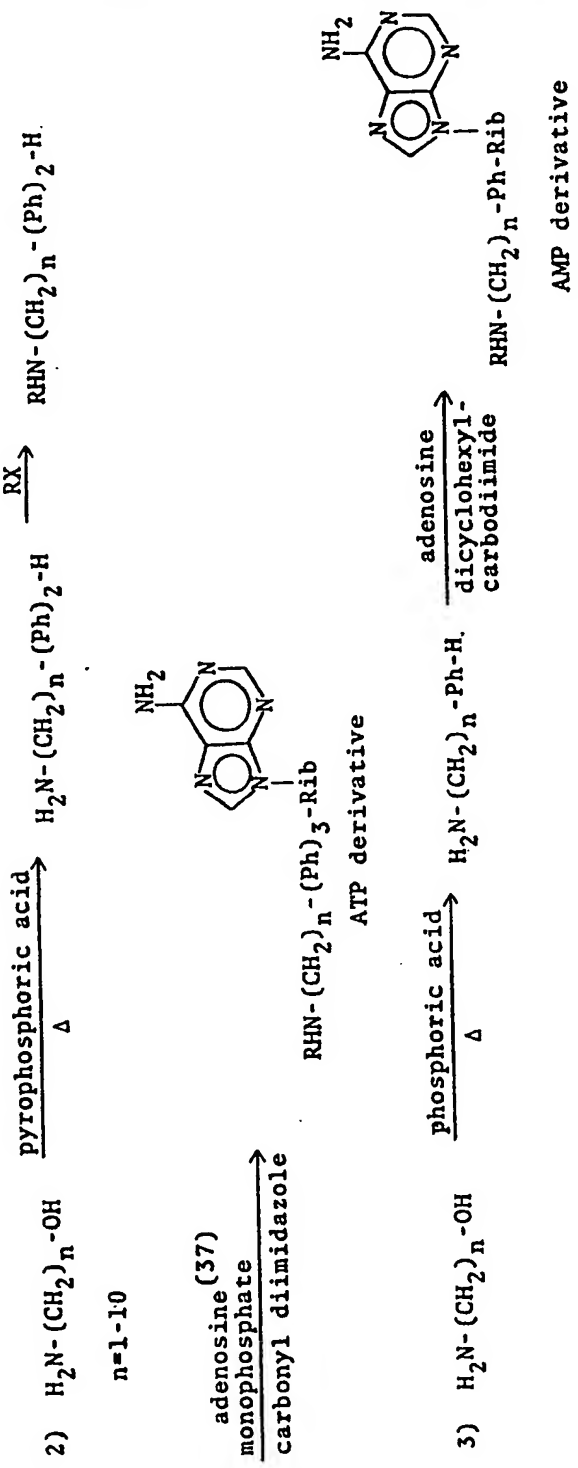
(36) Trayer, I.P., et al., *Biochem. J. 139*:609 (1974).



In one form of the present invention, the components of the specific binding reaction which are to be combined with the liquid medium suspected of containing the ligand are in a liquid or solid form. The assay method may be carried out in a standard laboratory vessel such as a test tube with the specific binding reaction components and the components of the reaction system being added thereto in solid or liquid form.

It is also contemplated that one or more of the specific binding reaction components and/or one or more of the components of the monitoring reaction may be

incorporated with a carrier. In one aspect, the carrier may be a liquid-holding vessel such as a test tube or capsule containing such component or components in an interior portion thereof, for instance, in the form of a liquid or loose solid or a coating on an interior surface of the vessel. In another aspect, the carrier may be in the form of matrix which is insoluble and porous, and preferably absorbent, relative to the liquid medium to be tested. Such matrix may be in the form of bibulous papers; polymeric films, membranes, fleeces, or blocks; and gels. In such a form, the liquid medium to be tested, for carrying out the specific binding reaction and/or the monitoring reaction, for effecting the necessary separation, and for observing the resulting response.

The liquid medium to be tested may be a naturally occurring or artificially formed liquid suspected of containing or known to contain the ligand, and usually is a biological fluid or a liquid resulting from a dilution or other treatment thereof. Biological fluids which may be assayed following the present method include serum, plasma, urine, and amniotic, cerebral, and spinal fluids. Other materials such as solid matter, for example tissue, or gases may be assayed by reducing them to a liquid form such as by dissolution of the solid or gas in a liquid or by liquid extraction of the solid.

In contrast to the prior art assay systems, biological fluids containing substances which have reactant activity similar or identical to that of the conjugated labeling substance may be assayed for the ligand without background interference. Endogenous background reactant activity can be readily eliminated in several manners. The biological fluid can be treated to selectively destroy the endogenous reactant activity. Such treatment may involve the action of a clearing agent which chemically destroys the endogenous activity followed by treatment to inactivate the destructive action of such clearing agent.

For instance, reactant-degrading enzymes often appear naturally in biological fluids, particularly if the reactant is a coenzyme such as NAD, NADP, or ATP. There are many inhibitors of such coenzyme-degrading enzymes, for example, chelating agents which operate to deprive the enzymes of essential metal ion activators. As a specific example, NAD-degradable enzymes are found in normal serum and have sufficient enzymatic activity to remove essentially all endogenous NAD activity from isolated serum within a few hours. The degrading activity of such enzymes may be effectively inhibited by addition of a chelating agent such as ethylenediamine tetraacetic acid. Elimination of the degrading activity may also be accomplished by adding a specific enzyme inhibitor. For example, ATP-degrading enzymes may be inhibited by addition of $\beta\gamma$ methylene ATP or $\alpha\beta$ methylene ATP.

The present invention will now be illustrated, but is not intended to be limited, by the following Examples.

EXAMPLE 1

Preparation of nicotinamide 6-(2-aminoethylamino)purine dinucleotide.

Two (2) grams of nicotinamide adenine dinucleotide (NAD) were dissolved in 10 ml of water and 0.6 ml of ethyleneimine was added dropwise, the pH being maintained below 7 by the addition of 1 M perchloric acid. When addition of ethyleneimine was complete, the pH was adjusted to 4.5 and the reaction was incubated at 20—25°C. At 24 hour intervals 0.6 ml of ethyleneimine was added and the pH readjusted to 4.5. After 96 hours, the solution was poured into 10 volumes of acetone at —10°C. The oil which formed was collected, washed with ether, and dissolved in approximately 50 ml of water in a flask.

The resulting solution was adjusted to pH 7.0—7.5 with 1 N sodium hydroxide, and 1 gram of sodium bicarbonate was added. Nitrogen was bubbled through the solution for from 4 to 5 minutes and 1 gram of sodium hydrosulfite was added. The flask was sealed tightly and allowed to stand at room temperature for 45 minutes. The solution was then oxygenated for 15 minutes and adjusted to pH 11.3 with sodium hydroxide. The solution was heated at 75°C for 1 hour. Then the reaction mixture was cooled to room temperature and 0.6 grams of tris-(hydroxymethyl)-aminomethane was added, followed by 5 N hydrochloric acid to adjust the pH to 7.5. To the resulting solution was added 1000 International units of alcohol dehydrogenase and 1 ml of acetaldehyde. The decreasing optical density of the reaction mixture was monitored at 340 nm and when no further decrease was observed, the pH was adjusted to 3.5. The solution was poured into 10 volumes of acetone at —10°C. The oil which formed was separated and washed with ether, after which it was dissolved in 10 to 15 ml of water.

The resulting solution was introduced into a 2.5×90 cm column of Sephadex G-10, available from Pharmacia AB, Uppsala, Sweden, equilibrated with water.

Fractions of 12 ml volume were collected. The wavelength of maximum optical absorption in the ultraviolet region and the optical density at such wavelength were determined for each fraction. Also, the optical density at 340 nm of each fraction after reduction with alcohol dehydrogenase was determined. The fractions which had an optical absorption maximum at 275 nm and had a ratio of optical density at 340 nm to that at 265 nm greater than 0.05 were pooled. The pooled material was concentrated to from 15 to 20 ml on a rotary evaporator and passed through a 2.5×28 cm column of Dowex 1-X8, available from Bio-Rad laboratories, Richmond, California, equilibrated with water. Additional water was added to wash the pooled material through the column, and 10 ml fractions were collected. The fractions which had an optical density at 340 nm to that at 264 nm greater than 0.1 were pooled.

The pooled material was passed through 5×45 cm column of Dowex 50-X2, available from BioRad Laboratories, Richmond, California, equilibrated with water. Additional water was added to wash the pooled material through the column and 20 ml fractions were collected. The fractions which had an optical absorption maximum at 264 nm and had a ratio of optical density at 340 nm to that at 265 nm greater than 0.18 were pooled. The pooled material was concentrated to from 4 to 5 ml and purified by electrophoresis as follows.

The concentrated material was applied to a sheet of Whatman 3MM paper, available from Reeve Angel, Clifton, New Jersey, in a 1 to 2 cm wide strip perpendicular to the direction of current flow. The paper was then wetted with 0.02 M sodium phosphate at pH 6.0. Electrophoresis was conducted according to the Durrum hanging paper method, as described in *Science 121*: 829 (1955), for 4—7 hours with a potential gradient of about 8.5 volts/cm. The location of the desired pyridine nucleotide derivative was determined by fluorescence developed after spraying a test strip of the paper with 0.5 M sodium cyanide according to the procedure described in *J. Biol. Chem. 191*: 447 (1951). The area containing the desired derivative was cut out of the paper and extracted with three (3) 50 ml volumes of water. The resulting extracts containing nicotinamide 6-(2-aminoethylamine) purine dinucleotide were pooled, concentrated to from 3 to 4 ml, and stored at —20°C.

EXAMPLE 2

Preparation of nicotinamide adenine dinucleotide-biotin conjugate.

A 16 mg quantity of biotin was suspended in 1 ml of water containing 22 mg of nicotinamide 6-(2-aminoethylamino) purine dinucleotide prepared as in Example 1. A few drops of 0.1 N sodium hydroxide was added to aid dissolution of the biotin. A 240 mg quantity of 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-p-tolulene sulfonate was added to the resulting solution and brought into solution by dropwise addition of 0.1 N hydrochloric acid. The reaction mixture was allowed to incubate at room temperature for 5 hours and was then poured into 10 ml of acetone at —10°C. The oil which formed was separated, washed twice with from 5 to 10 ml of ether and dissolved in from 1 to 2 ml of water. The resulting material was purified by electrophoresis on paper as in Example 1. Two fluorescent bands appeared after spraying with sodium cyanide, one having migrated toward the cathode and the other toward the anode. The latter band, which contained the NAD-biotin conjugate, was eluted with water and stored at —20°C.

EXAMPLE 3

Preparation of biotin-umbelliferone conjugate.

(2 - Oxo - 2 - H - 1 - benzopyran - 7 - yl) - 5 - [cis - hexahydro - 2 - oxo - 1H - thieno - (3,4 - d) - imidazole] valeric acid ester.

A solution of 300 mg (1.2 mmol) anhydrous biotin in 20 ml dry dimethylformamide was stirred at —10°C under dry nitrogen gas and 0.17 ml (1.2 mmol) dry triethylamine was added. A solution of freshly distilled ethyl chloroformate (0.141 ml in 3 ml of dry ether) was added dropwise. After incubation for 30 min with stirring, the resulting precipitate was filtered under a dry nitrogen atmosphere and cooled immediately to —10°C. To the filtered residue was added a solution of 197 mg (1.2 mmol) anhydrous 7-hydroxycoumarin in 3 ml dry pyridine and stirred for 1 hour at —10°C followed by 20 hours at 25°C. The solvents were evaporated under high vacuum at 40°C. After cooling, the resulting solid was filtered and recrystallized from methanol to yield the desired product (melting point=216—218°C).

Calculated for $C_{19}H_{20}N_2P_5S$:	C, 48.75; H, 4.19; N, 7.21.
Found:	C, 58.4; H, 5.12; N, 6.86.

EXAMPLE 4

Competitive binding-bioluminescence assay for biotin; effect of varying levels of biotin on the peak light intensity produced.

The bioluminescence reaction system used in this Example was based on the following reactions:

(a) NAD-ligand + ethanol $\xrightarrow[\text{dehydrogenase}]{\text{alcohol}}$ NADH-ligand + acetaldehyde

(b) NADH-ligand + FMN* + $H^{\oplus}$ $\xrightarrow[\text{dehydrogenase}]{\text{NADH}}$ NAD-ligand + $FMNH_2$

(c) $FMNH_2$ + long-chain aldehyde + O_2 $\xrightarrow{\text{luciferase}}$ FMN + long-chain acid + H_2O + $h\nu$

* flavin mononucleotide

A. Preparation of light-generating solution

A light-generating solution for carrying out reactions (b) and (c) was prepared as follows. A reagent mixture was prepared containing 0.13 M phosphate buffer at pH 7.0, 0.67 wt % bovine serum albumin, 15.7 μM flavin mononucleotide (FMN), and 13.3 mM sodium acetate, and this mixture was stored in the dark at −20°C. An emulsion of 5 μl of dodecanal in 5 ml of water was prepared the day the light-generating solution was to be used. Lyophilized luciferase extracted from *Photobacterium fisheri* (enzyme available from Worthington Biochemical Corp., Freehold, New Jersey) was added to 0.013 M phosphate buffer at pH 7.3 to a concentration of 20 mg/ml. After 30 minutes the resulting suspension was centrifuged at 1500 xg for 10 minutes and the pellet was discarded. The light-generating solution was then prepared within 5 minutes of use by combining 75 μl of the reagent mixture, 5 μl of the dodecanal emulsion, and 20 μl of the luciferase solution.

B. Preparation of insolubilized binding partner.

Avidin, which has a binding affinity for biotin, was insolubilized by being covalently bound to a water insoluble polymer bead as follows. A quantity of Sepharose 4B (available from Pharmacia AB, Uppsala, Sweden) was activated for bonding to avidin using the method of March et al, *Analytical Biochemistry 60*: 149 (1974). Approximately 4 ml of the activated Sepharose 4B was suspended in 8 ml of 0.1 M citrate buffer at pH 7.0. To the suspension was added 6 mg of avidin, having an activity of 9.9 units/mg, in 3 ml of water. One unit of avidin activity is that quantity of avidin capable of binding 1 μg of biotin. The resulting reaction mixture was stirred for 6 hours at 7°C. The avidin-bound-Sepharose 4B was then filtered, washed with 100 ml of 0.1 M sodium bicarbonate buffer at pH 9.0, and resuspended in 240 ml of 0.1 M tris-(hydroxymethyl)aminomethane hydrochloride buffer at pH 8.0.

C. Control experiments

Nine specific binding reaction mixtures were prepared, each having a total volume of 0.19 ml and each containing 0.1 M tris-(hydroxymethyl)-aminomethane hydrochloride buffer at pH 8.0, 0.6 M ethanol, 0.01 M semicarbazide hydrochloride, and respectively the amounts or concentrations indicated in Table 1 of NAD, NAD-biotin conjugate, avidin-bound Sepharose 4B suspension (prepared according to Part B of this Example), and Sepharose 4B suspension (formed by suspending 1 ml of packed Sepharose 4B in 60 ml of 0.1 M tris-(hydroxyethyl)-aminomethane hydrochloride buffer at pH 8.0). The reaction mixtures were shaken gently for 15 minutes at room temperature. Then, 0.22 International units of alcohol dehydrogenase was added to each reaction mixture to initiate the reduction reaction. Semicarbazide combines with the acetaldehyde produced in reaction (a) to form a semicarbazone and thus to drive reaction (a) in the desired direction.

The reaction mixtures were shaken again for 15 minutes at room temperature.

A 10 μl aliquot of the supernatant from each reaction mixture was then injected into a separate cuvette mounted in a DuPont Model 760 Bioluminescence Photometer (E. I. duPont de Nemours, Willmington, Delaware) containing 100 μl of the previously prepared light-generating solution which had been pre-incubated for from 2 to 3 minutes at 25°C. The results appear in Table 1.

TABLE 1

reaction	concentration of NAD (nM)	concentration of NAD-biotin conjugate (nM)	avidin-bound Sepharose 4B suspension (μl)	Sepharose 4B suspension (μl)	peak light intensity
1	—	—	—	—	1.2
2	21	—	—	—	159
3	21	—	20	—	147
4	—	10	—	—	45.9
5	—	21	—	—	110
6	—	21	20	—	28.5
7	21	—	—	10	154
8	—	21	—	10	114
9	—	—	20	—	1.6

The results of control reactions 1 and 9 show that in the absence of NAD and NAD-biotin conjugate very little light was produced. Reactions 2 and 3 yielded results indicating that the light producing reaction occurred when free NAD was added and that such reaction was substantially unaffected by the presence of avidin-bound-Sepharose 4B. The results of reactions 4, 5, and 6 show that the NAD-biotin conjugate was active in the light producing reaction, that the peak light intensity produced increased as more NAD-biotin conjugate was present, and the presence of avidin-bound-Sepharose 4B inhibited light production. Comparison of the results of reactions 3 and 5 with those of 7 and 8 shows that the light producing reaction was not affected by the presence of plain Sepharose 4B.

D. Assay method

Five additional specific binding reaction mixtures were prepared, each having a volume of 0.19 ml and each containing 0.1 M tris-(hydroxymethyl)-aminomethane hydrochloride buffer at pH 8.0, 0.6 M ethanol and 0.01 M semicarbazide, and respectively the amounts or concentrations indicated in Table 2 of NAD-biotin conjugate, free biotin, and avidin-bound-Sepharose 4B suspension. Each reaction mixture was treated in the same manner as the control reaction mixtures in Part C of this Example. The results appear in Table 2.

TABLE 2

reaction	concentration of NAD-biotin conjugate (nM)	concentration of biotin (nM)	avidin-bound Sepharose 4B suspension (μl)	peak light intensity
10	21	—	—	79.1
11	21	—	20	17.4
12	21	79	20	43.1
13	21	158	20	59.9
14	21	158	—	79.7

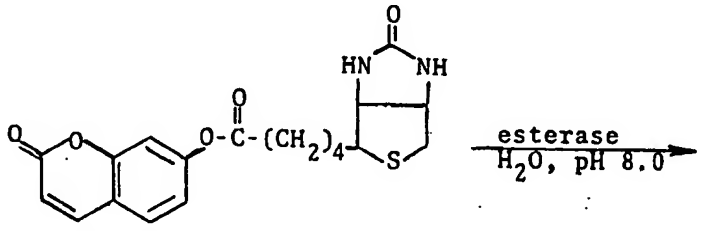
The results of reactions 11, 12, and 13 show that free biotin and NAD-biotin conjugate compete effectively for the binding sites on the insolubilized avidin since the peak light intensity produced was dependent upon the amount of free biotin present. Reactions 10 and 14 gave results indicating that in the absence of insolubilized avidin, the peak light intensity produced was constant for vastly different concentrations of free biotin.

It was thus demonstrated in this Example that the amount of NAD-biotin conjugate in the liquid phase was inversely related to the amount of free biotin present and thus the assay method and means of the present invention are useful in the determination of a ligand in an unknown liquid sample.

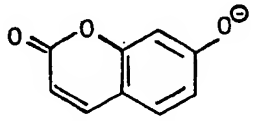
EXAMPLE 5

Specific binding assays for avidin and biotin employing an enzyme substrate as labeling substance.

The specific binding assay systems used in this Example were based on the following reaction:



biotin-umbelliferone conjugate



$+ H^{\oplus}$ + biotin

(max. fluorescence at 448 nm)

A. Preparation of insolubilized binding partner.

Avidin was insolubilized by being covalently bound to a water insoluble polymer bead as in Part B of Example 4 except that after washing with 100 ml of 0.1 M sodium bicarbonate buffer at pH 9.0, the avidin-bound-Sepharose 4B was suspended in 12 ml of 0.1 M tris-(hydroxymethyl)aminomethane hydrochloride buffer at pH 8.0 and diluted 1:1 with 0.1 M bis-hydroxyethylglycine hydrochloride buffer at pH 7.0.

B. Competitive binding assay of biotin; effect of various levels of biotin on the amount of umbelliferone liberated.

Eight specific binding reaction mixtures were prepared, each having a total volume of 0.2 ml and each containing 0.1 M bis-hydroxyethylglycine hydrochloride buffer at pH 7.0, 0.3 μM biotin-umbelliferone conjugate (prepared as in Example 3), 15 μl of the avidin-bound-Sepharose 4B suspension prepared as in Part A of this Example, and biotin in the concentrations indicated in Table 3. The reaction mixtures were allowed to incubate at room temperature with gentle shaking for 20 minutes. Each reaction mixture was centrifuged and a 100 μl aliquot of the supernatant was combined with 2 ml of 0.1 M bis-hydroxyethylglycine hydrochloride buffer at pH 8.2 containing 1.08 units of porcine esterase. After a 5 minute incubation at room temperature, the fluorescence intensity produced in each reaction mixture at 448 nm with excitation at 364 nm was measured using an Amico-Bowman spectrophotofluometer. The results appear in Table 3.

TABLE 3

reaction mixture	concentration of biotin (μM)	fluorescence intensity
1	0.00	0.355
2	0.10	0.495
3	0.20	0.469
4	0.30	0.503
5	0.40	0.547
6	0.50	0.502
7	0.75	0.580
8	1.00	0.688

It was thus demonstrated in this Example that the amount of NAD-biotin in the liquid phase was directly proportional to the amount of free biotin present and thus the assay method and means of the present method are useful in the determination of a ligand in an unknown liquid sample.

The words "Sephadex", "Dowen", "Whatman", and "Sepharose" are registered Trade Marks.

WHAT WE CLAIM IS:—

1. A heterogenous specific binding method for assaying a liquid medium for a ligand, which comprises:

(a) contacting the said liquid medium with reagent means comprising a labelled conjugate of (1) a labelling substance having a predetermined activity as (i) a substrate in an enzyme-catalysed chemical reaction, (ii) a reactant in a cyclic chemical reaction, (iii) a reactant in a chemiluminescent reaction, or (iv) a coenzyme in a chemical reaction, and (2) a binding component, the said reagent means forming with the said ligand in a binding reaction system so as to produce a bound-phase in which the binding component of the said labelled conjugate is bound by a specific binding partner therefor and a free-phase in which the binding component of the labelled conjugate is not bound by a specific binding partner therefor, the amount of the predetermined characteristic of the said labelling substance in said bound-phase or the said free phase being a function of the amount of said ligand present in said liquid medium under assay;

(b) separating the said bound-phase from the said free-phase; and

(c) determining the quantity of the said labelling substance present in either the said bound-phase or the said free-phase, by respectively (i) adding the said enzyme thereto and measuring the substrate activity of the labelling substance therein, (ii) forming the said cyclic chemical reaction therein and measuring the said activity, (iii) forming said chemiluminescent reaction therein and measuring the light produced, or (iv) forming the enzyme reaction therein and measuring the said coenzyme activity.

2. A method as claimed in Claim 1 wherein the labelling substance has predetermined activity as a substrate in an enzyme-catalysed chemical reaction, the said conjugate includes a bridge group through which the labelling substance is coupled to the binding component, and the said enzyme catalyses the cleavage of the said bridge group to release a detectable molecule.

3. A method as claimed in Claim 2 wherein the said bridge group is an ester group and the said enzyme is an esterase.

4. A method as claimed in Claim 2 or 3 wherein the said released detectable molecule is fluorescent.

5. A method as claimed in Claim 3 or 4 wherein the said released fluorescent molecule is umbelliferone or fluorescein, or a derivative thereof.

6. A method as claimed in Claim 1 wherein the labelling substance has predetermined activity as a substrate in an enzyme-catalysed chemical reaction and the said enzyme acts on the said conjugate to form a product which has a detectable property which distinguishes it from the said conjugate.

7. A method as claimed in Claim 6 wherein the said distinguishing property is a fluorescent property.

8. A method as claimed in Claim 1 wherein the labelling substance has predetermined activity as a reactant in an autocatalytic cyclic chemical reaction.

9. A method as claimed in Claim 1 or 8 wherein the said labelling substance has the activity of a material which is cycled in a said cyclic chemical reaction.

10. A method as claimed in Claim 1 wherein the labelling substance has predetermined activity as a reactant in a chemiluminescent reaction and the light produced by the chemiluminescent reaction is measured as the total amount of light produced.

11. A method as claimed in Claim 1 wherein the labelling substance has predetermined activity as a reactant in a chemiluminescent reaction and the light produced by the chemiluminescent reaction is measured as the peak intensity of light produced.

12. A method as claimed in Claim 1, 10 or 11, wherein the said labelling substance has the activity of luminol, isoluminol, or a derivative thereof, in a chemiluminescent reaction.

13. A method as claimed in Claim 1 wherein the labelling substance has predetermined activity as a nucleotide coenzyme in a chemical reaction.

14. A method as claimed in Claim 1 or 13 wherein the labelling substance has predetermined activity as an adenosine phosphate, nicotinamide adenine dinucleotide or a reduced form thereof, or nicotinamide adenine dinucleotide phosphate or a reduced form thereof.

15. A method as claimed in Claim 14 wherein the labelling substance has the activity of adenosine triphosphate or flavin adenine dinucleotide in said chemical reaction.

16. A method as claimed in any one of Claims 1 to 15 wherein the character of said predetermined activity of the said labelling substance in the labelled conjugate in the bound-phase is substantially the same as in the free-phase.

17. A method as claimed in any one of Claims 1 to 16 wherein the said ligand is

an antigen or antibody thereto; a hapten or antibody thereto; or a hormone, vitamin, metabolite, or pharmacological agent, or a receptor or binding substance therefor.

18. A method as claimed in any one of Claims 1 to 17 wherein the said liquid medium is a biological fluid.

19. A test system for use in a heterogenous specific binding method for assaying a liquid medium for a ligand, which comprises a labelled conjugate of (1) a labelling substance having a predetermined activity as (i) a substrate in an enzyme-catalysed chemical reaction, (ii) a reactant in a cyclic chemical reaction, (iii) a reactant in a chemiluminescent reaction, or (iv) a coenzyme in a chemical reaction, and (2) a binding component, the said test system forming with the said ligand a binding reaction system so as to produce a bound-phase in which the binding component of the said labelled conjugate is bound by a specific binding partner therefor and a free-phase in which the binding component of the labelled conjugate is not bound by a specific binding partner therefor, the amount of the predetermined characteristic of the said labelling substance in said bound-phase or the said free phase being a function of the amount of said ligand present in said liquid medium under assay; and also, when the said labelling substance has a predetermined activity as a substrate in an enzyme-catalysed reaction, the said enzyme.

20. A test system as claimed in Claim 19 wherein the labelling substance has predetermined activity as a substrate in an enzyme-catalysed chemical reaction, the conjugate includes a bridge group through which the labelling substance is coupled to the binding component, and the said enzyme catalysed the cleavage of the said bridge group to release a detectable molecule.

21. A test system as claimed in Claim 20 wherein the said bridge group is an ester group and the said enzyme is an esterase.

22. A test system as claimed in Claim 20 or 21 wherein the said released detectable molecule is fluorescent.

23. A test system as claimed in Claim 22 wherein the said released fluorescent molecule is umbelliferone or fluorescein, or a derivative thereof.

24. A test system as claimed in Claim 19 wherein the labelling substance has predetermined activity as a substrate in an enzyme-catalysed chemical reaction and the said enzyme acts on the said conjugate to form a product which has a detectable property which distinguishes it from the said conjugate.

25. A test system as claimed in Claim 24 wherein the said distinguishing property is a fluorescent property.

26. A test system as claimed in Claim 19 wherein the labelling substance has predetermined activity as a reactant in an autocatalytic cyclic chemical reaction.

27. A test system as claimed in Claim 19 or 26 wherein the labelling substance has the activity of a material which is cycled in a said cyclic chemical reaction.

28. A test system as claimed in Claim 19 wherein the said labelling substance has the activity of luminol, isoluminol, or a derivative thereof, in a chemiluminescent reaction.

29. A test system as claimed in Claim 19 wherein the labelling substance has predetermined activity as a nucleotide coenzyme in a chemical reaction.

30. A test system as claimed in Claim 19 or 29, wherein the labelling substance has predetermined activity as an adenosine phosphate, nicotinamide adenine dinucleotide or a reduced form thereof, or nicotinamide adenine dinucleotide phosphate or a reduced form thereof.

31. A test system as claimed in Claim 30 wherein the labelling substance has the activity of adenosine triphosphate or flavin adenine dinucleotide in said chemical reaction.

32. A test system as claimed in any of Claims 18 to 31, wherein one or more of the constituents are in a dry form.

33. A test system as claimed in any of Claims 18 to 31, wherein one or more of the constituents are incorporated in a carrier.

34. A test system as claimed in Claim 33 wherein said carrier is absorbent relative to said liquid medium.

35. A method as claimed in Claim 1 substantially as described in any one of the foregoing Examples.

36. A test system as claimed in Claim 19 substantially as described in any one of the foregoing Examples.

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